THE ABSORPTION AND METABOLISM OF 2,4-DICHLOROPHENOXYACETIC ACID BY CITRUS LEAVES

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INTRODUCTION

As the case with many tree fruits, the preharvest drop of citrus fruits is a serious problem, particularly in certain years. In searching for agents to prevent preharvest drop, it was found that 2,4-dichlorophenoxyacetic acid (2,4-D) would delay abscission of fruits on certain varieties (10, 20, 24, 26, 28, 43, 52, 60, 61, 62, 70) and not on others (10, 71, 74). Moreover, the response of a particularly variety varied with the geographical location (71, 74) and from year to year (57). Recently, it has been shown with tests of 2,4-D at low concentration on explants of several varieties that they all responded to 2,4-D, even varieties previously found to respond very little to field application of 2,4-D (92). The difference in the response to the auxin could be due to a difference in uptake, translocation, metabolism of 2,4-D per se, and/or tissue responses to 2,4-D.

Therefore, the objectives of this investigation were to determine the patterns of uptake, translocation, and metabolism of 2,4-D by a variety of citrus that responds to low concentrations of the chemical and a variety that does not respond. Furthermore, since plant responses have been found to be due to free 2,4-D in the tissues (87, 89), several indicators of metabolism, CO_2 evolution, O_2 uptake and ratio of glucose-1-^{14}C and glucose-6-^{14}C metabolism, were used in an attempt to determine tissue sensitivity to 2,4-D. It was anticipated that a knowledge of these patterns in a susceptible and a non-susceptible variety would help to determine the rate of 2,4-D in preventing fruit abscission.

LITERATURE REVIEW

There have been many reports of the use of 2,4-dichlorophenoxyacetic acid (2,4-D) as an auxin or as a herbicide (14, 16, 36, 67, 84, 85, 86).

This review covers primarily the uptake, absorption and metabolism of 2,4-D and its effects on abscission.

Effects of 2,4-D on Abscission

Previous reports reveal that there has been considerable use of 2,4-D and other carboxylic acids for the prevention of the abscission of plant organs. In 1945, Batjer and Marth (9), while searching for materials to delay abscission of apples, found that 2,4-D was effective over a period nearly twice as long as that of α-naphthalene-acetic acid (NAA). Later, it was demonstrated that there was a high degree of varietal selectivity when 2,4-D was used to control apple drop, being effective on 'Winesap' but not on others tested (10). Harley et al. (43) also applied 2,4-D to reduce preharvest fruit drop of apples and found that even weak concentrations (0.001%) were effective in preventing fruit drop in one variety, but was ineffective in others. They did not give any explanation for such specificity. Others (25, 27, 65) observed that 2,4-D was effective in reducing abscission of 'Stayman' and 'Winesap' apples plus demonstrating that other apple varieties would respond to 2,4-D. It was also noted with 'Stayman' and 'Winesap' that leaves tended to remain greener and persist longer on trees sprayed with 2,4-D (27).

While working with certain factors affecting the inhibition of abscission of apple pedicels or petioles by NAA and 2,4-D, Barlow (6) found a linear relation between concentration of the growth substance and persistence of the organ. Later, it was demonstrated (28) that 2,4-D was more effective than NAA in delaying petiole abscission of early 'McIntosh' and 'Northern Spy' apple varieties, and that 2,4-D was also effective in delaying abscission of preharvest fruits of early 'McIntosh'.

Fruit abscission can be prevented on some citrus varieties with 2,4-D. When 2,4-D was used as an amendment to oil sprays and applied to 'Valencia', 'Washington' navel oranges, 'Mediterranean Sweet' oranges and lemons, it was found that 2,4-D limited leaf and fruit drop. Also, there was an increase in the storage life of lemons and grapefruit, as a result of the 2,4-D application (76, 78). In the case of the latter, the treatment presumably prevented a high incidence of rot at the stem end of the fruit by preventing the abscission of the 'button', a name applied to the adhering stem piece. The physiological effect was shown not only by the maintenance of the button in a green stage, but also by the delay in fruit maturity. In other experiments (29, 77, 79, 80, 82), 2,4-D was found to reduce preharvest fruit drop, and to increase fruit size on 'Valencia' and 'Washington' navel oranges. This size increase was primarily due to an increase in the growth rate of the immature fruit.

The use of 2,4-D over a wide range of conditions was found (21, 81) to be effective in the reduction of mature fruit drop on 'Washington' navel oranges in both California and New Zealand. In Florida (74), 2,4-D and the triethanolamine salt of 2,4,5, trichlorophenoxypropionic acid (2,4,5-TP) reduced the fruit lost due to preharvest drop of

'Pineapple' orange and was found then that 2,4-D and 2,4,5-TP were fairly specific for 'Pineapple' oranges.

Studies (40) of the effects of various compounds on preharvest fruit drop of citrus under Florida conditions showed that 2-methyl, 4 chlorophenoxy-acetic acid, 2 methylphenoxy alpha-butyric and 2-methyl-4-bromophenoxyacetic acid had some influence on controlling the drop of both 'Pineapple' and 'Valencia' oranges. Others (71) demonstrated that 2,4-D exerted an appreciable delaying effect on preharvest fruit drop of 'Pineapple' but not of 'Valencia'. These results contradicted those reported from California with 'Valencia' (76). Moreover, 2,4-D was reasonably effective in preventing fruit drop of sweet orange seedlings and 'Temple' oranges but had no effect on 'Marsh' grapefruit (71).

With leaves, 2,4-D has an inhibitory effect on abscission. Livingston (59) employed fully mature leaves of 'Valencia' orange as explants for an _in vitro_ test of abscission agents. His results showed that even the lowest concentration of 2,4-D (10^{-4} M) had an inhibitory effect on abscission. Chaudri (19) also found an abscission-inhibiting effect of 2,4-D in test with 'Hamlin' orange explants. Day and Erickson (23) reported the ring-substituted mono-, di-, and trichlorophenoxyacetic acids were tested for their activity in preventing ethylene-induced abscission on leaves from lemon cuttings. They found that maximum activity was associated with compounds having chlorines in the 2nd and 4th positions and hydrogen in the 6th position. An additional chlorine atom in either the 3rd or 6th position had no effect on the activity of the phenoxyacetate derivatives.

Studies of the influence of indole-3-acetic acid (IAA), NAA and various phenoxyacetic acids on abscission of leaves of Phaseolus vulgaris L. showed the compounds tested inhibited abscission, and that there was a general effectiveness of the phenoxy compounds on inhibition of abscission, with those compounds possessing the greatest auxin activity (like 2,4-D or 2,4,5-T) also producing the strongest inhibition (18). Wilson and Hendershott (92) screened many chemicals and chemical combinations in an attempt to induce abscission of 'Hamlin' orange explants. They found that 2,4-D at low concentrations (10^{-7} M) increased abscission, but high concentrations (10^{-4} M) delayed abscission considerably.

Absorption and Translocation of 2,4-D

It has been established that 2,4-D is absorbed, translocated and distributed through leaves and stems of different plant species.

Studies of the distribution of a carbon-^{14}labeled 2,4-D in bean plants at various times after application to the leaves demonstrated a small amount of radioactivity in different parts of bean plants as early as 2 hours after treatment with 2,4-D-^{14}C. The principal movement of 2,4-D-^{14}C was downward in the stems, eventually reaching the roots (48). This work indicated that 2,4-D was quickly absorbed and the ^{14}C has translocated to the untreated leaf, root and terminal bud. Young plants absorbed 2,4-D and translocated ^{14}C at a faster rate than older plants. Also a small amount of 2,4-D was found to be metabolized in the bean plant with the subsequent loss of $^{14}CO_2$.

In contrast to this, Fang (30), working with the absorption, translocation and metabolism of 2,4-D-1-^{14}C in pea and tomato plants, found pea plants only absorb 2,4-D the first day after application to the leaves, while tomato leaves continuously absorbed the applied 2,4-D throughout the entire 7 days of the experiment. In relation to absorption and translocation, Day (22) reported that 2,4-D moved through the cuticle, epidermis and mesophyll and into the phloem of beans with a velocity varying through a range of 10 to 100 cm per hour. In a study of the absorption and translocation of 2,4-D-1-^{14}C by corn and wheat plants, it was found that wheat and corn absorbed 2,4-D at a slower rate than did bean plants (32). Also, translocation of ^{14}C to the apical portion of the corn and wheat plants was very slow. Moreover, Gallup and Gustafson (39), when working on the absorption and translocation of 2,4-dichloro-5-^{131}Iodo-phenoxyacetic acid in a number of mono- and dicotyledonous plants, found that this growth regulator was absorbed by all the species used, but monocotyledonous plants absorbed it at a slower rate than dicotyledonous plants. Translocation to the apical portion of the plant was very rapid in dicots and very slow in the grasses. The question of how 2,4-D exerts its selective herbicidal action on monocotyledonous and dicotyledonous plants has not been completely answered, but the slower absorption and distribution rates and faster detoxification reactions within monocots are believed to be contributing factors in their greater resistance to 2,4-D (32, 39, 44).

A method was developed for the extraction and bioassay of 2,4-D from treated plants (45, 46). It was found the transport of 2,4-D ceases at about the middle of the hypocotyl of bean seedlings and none entered the roots at any time; this was in contrast to the previous demonstration by Holley *et al*. (48) that 2,4-D moved freely to the roots.

Transport has been demonstrated to be proportional to the amount of 2,4-D applied, up to 75 mg per plant; greater amounts inhibited transport out of the leaf (45). Also, transport of 2,4-D from leaves in the dark did not take place, but could be induced by applying sucrose to the leaves (46, 54). Application of sucrose to leaves in the light did not increase the transport of 2,4-D. They reasoned that transport of 2,4-D was not due to simple osmotic forces as in the mass flow hypothesis, but involved a metabolic component. In contrast to this, Barrier and Loomis (8) found that absorption alone of 2,4-D by pea leaves required less than 2 hours, and was increased by surface-active chemicals, and by increased temperature. It was not reduced by depletion of carbohydrates.

Studying the effects of temperature and humidity on foliar absorption and translocation of 2,4-D, Pallas (69) found that absorption and translocation increased with increasing temperatures from 20° to 30° C., but at such temperatures, less 2,4-D was absorbed and translocated at low humidities (34-48%) than at high humidities (70-74%). The movement of 2,4-D in the pea leaves was confined in general to the vascular bundles and followed the route of the assimilate stream from the leaf. He also reported that the bulk of radioactivity was being transported as free 2,4-D.

Jacobs _et al_. (53) working with _Phaseolus vulgaris_, measured the transport of 2,4-D through the abscission zones, pulvini and petioles, of young and old primary leaves. They found that the pulvinus limited the basipetal movement of 2,4-D through explants from old leaves, which included both pulvinar and petiolar tissue, but not in explants from young leaves. There was no evidence that the abscission zone itself was a barrier to 2,4-D transport. In earlier reports (63,64) it had been demonstrated that the movement of radioactive IAA and 2,4-D in segments from petioles of Phaseolus vulgaris was by active polar transport.

Sargent (72) and Sargent and Blackman (73) investigated the complexity of factors controlling the entry of 2,4-D into leaves of Phaseolus vulgaris and Coleus. They concluded that both physical (age of the leaves, temperature, surface-active agent added), and metabolic factors influenced the rate of penetration of 2,4-D into the leaf.

Influence of 2,4-D on Metabolism

Many reports have been published on the influence of 2,4-D on respiration, especially when 2,4-D was used at herbicidal concentrations (2, 55, 56, 75).

In general, it was found (38, 55, 56) that there was an increase in the rate of respiration induced by 2,4-D treatments. Also, it was shown (37) that 2,4-D decreased the rate of photosynthesis with higher concentrations being more effective. It was stated (87) that the inhibition of photosynthesis by 2,4-D was related to the concentration of undissociated acid molecules in the solution used for treatment.

Several workers (7, 11, 24, 41) have presented evidence that a sequence of glucose breakdown other than that of glycolysis by the Embden-Meyerhof-Parnas pathway (EMP) plays an important part in the respiration of plant tissues. Later, Gibbs and Beevers (42) concluded that in a wide variety of higher plants the carbohydrate metabolism undergoes a qualitative change as the plant develops. In general, glucose catabolism is via the EMP pathway in young and meristematic tissues; but as the tissues age, the contribution of the Direct Oxidation pathway (PPP) of glucose metabolism increased (42).

The method used to evaluate the relative contributions of the EMP sequence and the PPP essentially involves the measurement and comparisons of radiochemical yield in the CO_2 produced by equal samples of tissue respiring glucose-1-^{14}C and glucose-6-^{14}C, respectively (11).

Humphreys and Dugger (49, 50) reported that etiolated pea seedlings (cotyledons removed) were able to maintain higher rates of respiration after treatment with 2,4-D. They also showed that carbohydrate was the major substrate being oxidized in both treated and untreated seedlings. They concluded that the higher rate of respiration exhibited by the 2,4-D treated seedlings was not due to a greater amount of respiratory substrate present in the seedlings but to an increase in the amount of glucose metabolized via the PPP in root tips of 2,4-D treated pea, corn and oat seedlings. Working with explants of corn roots, they (51) further demonstrated that 2,4-D treatment inhibited the uptake of glucose but there was almost a doubling of $^{14}CO_2$ production. They concluded that this increase in CO_2 production, when 2,4-D treated roots were used, was due to a blocking of "synthetic" metabolic pathways by 2,4-D.

In contrast to the above, Fang et al. (33) studied the effect of 2,4-D upon the absorption and metabolism of glucose in bean stem and found that the uptake of glucose by 2,4-D treated stems was greatly increased. They concluded that 2,4-D may have a different function of modifying the biochemical processes in different plant tissues as well as tissues from different plant species. They also postulated that 2,4-D treatments did not affect the synthetic pathway for cellular constituents in the bean stem tissue.

Black and Humphreys (13) investigated the mechanisms whereby 2,4-D shifted the normal pathway of glucose catabolism. In this study, they showed that glycolytic enzymes, and several enzymes of the pentose phosphate cycle, were present in about the same quantities in extracts from roots of both phosphate buffer treated and 2,4-D treated corn seedlings. These *in vitro* studies did support the *in vivo* observation that 2,4-D treatment of etiolated corn seedlings affected glucose catabolism through an increase in the amount catabolized via the PPP.

Also, investigating the possible relationship between the susceptibility of plants to 2,4-D treatment and the effect on the pathways of glucose metabolism, Fang (31) found the magnitude of reduction in the ratio between anabolic and catabolic utilization of glucose, as found in the bean tissue from 2,4-D treated bean plants, was not observed in the stem tissues of other species (cucumber, pea, tomato, and corn). In the case of the bean tissues, he suggested that the decrease in the amount of glucose catabolized via the PPP in 2,4-D treated bean stems was due neither to a limitation of the enzyme concentration nor to an inhibition of the enzymic process.

Metabolism of 2,4-D by Tissues

Since plants respond quite differently to 2,4-D, investigations have been made of the metabolism *per se* of the compound in plants.

Fang (30); and Fang and Butts (32) found 2 major compounds designated as unknown 1 and unknown 3, arising from 2,4-D in treated plants, present in an 80% ethanol extract. Jaworski and Butts (54), studying the metabolism of ^{14}C labeled 2,4-D in bean plants, found 3 mojor radioactive compounds in an 80% alcohol extracts of bean stems from plants treated with either 2,4-D-1-^{14}C or 2,4-D-2-^{14}C. One of the compounds was identified as free 2,4-D. The concentration of 2,4-D in the stem extracts decreased to a minimum in about 14 days and then increased. The concentration of unknown 1 increased when 2,4-D decreased, and there was a drop in its concentration at the time the concentration of 2,4-D began to increase. They suggested that a detoxification reaction may be operative in the plant, and that unknown 1 may be a glycoside containing 2,4-D as the aglycon group. They did not identify unknown 2 but stated it was water soluble and ether insoluble. Others have (47, 48) applied 2,4-D-1-^{14}C to bean plants, and 1 week later found 60% of the radioactivity present as a water-soluble radioactive material. Holley (47) working with this unknown compound found it to be dialyzable, and that it could be hydrolyzed by acid or alkali to an ether extractable organic acid. He suggested that the unknown radioactive acid may be 3, 5, or 6-hydroxy-2-4-dichlorophenoxyacetic acid. He postulated that the unknown may have no relation to the action of 2,4-D and may be simple a detoxification product, although he did not rule out the possibility that it could actually be the active agent.

Weintraub et al. (90, 91) applied radioactive 2,4-D to bean plants, and found that $^{14}CO_2$ was produced from either 2,4-D-1-^{14}C or 2,4-D-2-^{14}C but the initial rate of production from 2,4-D-1-^{14}C was several times faster than from 2,4-D-2-^{14}C. No $^{14}CO_2$ was evolved when 2,4-D labeled in carbon one position of the ring was applied. It was also reported that the applied 2,4-D-^{14}C labeled in the side chain was transformed and distributed in compounds such as: organic acids, sugars, starch, pectin, protein, and cell wall substances.

Working with black currant, apple, strawberry and *Syringa vulgaris*, Luckwill and Lloyd-Jones (60, 61), and Edgerton (28) found that the rate of decarboxylation was always higher in varieties resistant to 2,4-D. In contrast to this, Morgan and Hall (66) applied 2,4-D-1-^{14}C to cotton and sorghum, and found that cotton decarboxylated the side-chain of 2,4-D several times faster than sorghum. Cotton is susceptible to 2,4-D and sorghum fairly resistant.

Bach (3) isolated radioactive metabolites of 2,4-D-1-^{14}C from bean stems. He found that one-half of the original radioactivity was in 10 compounds extractable with ether; that these components retained the aromatic nucleus of 2,4-D, and that neither 2,4-D nor 6- hydroxy- 2,4-D was present in the extracts. Later, Bach and Fellig (5) reported that there was a correlation between the inactivation of 2,4-D, and the cessation of callus growth in bean stem sections. The carboxyl labeled 2,4-D remained in the tissue in an ethanol-soluble form chromatographically distinct from 2,4-D and only relatively small amounts of radioactivity were found in the CO_2 evolved.

MATERIALS AND METHODS

The plant material used in these studies consisted of 'Pineapple' and 'Valencia' orange leaves from 8-year-old trees growing in experimental plantings at the University of Florida; and of 2-year-old 'Pineapple' and 'Valencia' trees grown in 6-inch pots in the greenhouse. The potted plants were used for studies where the leaves were treated while still attached to the plant.

For some of the studies, small mature branches were taken from the orchard trees and brought to the laboratory in enclosed plastic bags. Leaves were washed very carefully and the petioles cut under water. The detached leaves were used immediately in the tests.

Techniques for Studies of Uptake

The compounds used were 2,4-dichlorophenoxyacetic acid (2,4-D) labeled with carbon-14 in the carboxyl and methyl position (2,4-D-1-^{14}C and 2,4-D-2-^{14}C), obtained from Nuclear Equipment Chemical Corp., Farmingdale, N. Y. A sample of 7.3 mg of 2,4-D-1-^{14}C contained 0.1 mc of ^{14}C (specific activity, 3.03 mc/m mole, and 20.1 mg of 2,4-D-2-^{14}C contained 0.1 mc of ^{14}C (specific activity 1.0 mc/m mole).

For the studies on uptake, solutions of 2,4-D were prepared from these radioactive materials plus enough unlabeled 2,4-D to make 10^{-3} M stock solutions (specific activity 0.22 and 0.19 mc/m mole, respectively).

Absorption Through Leaf Surfaces Using Droplets

For the tests of uptake through leaf surfaces, plants were placed in a fume hood in the laboratory immediately after removal from the greenhouse. The 2,4-D-^{14}C solution (10^{-3} M) was applied on the upper surface of intact leaves by placing a 10 μl drop on the lamina on both sides of the midrib approximately one-half the distance from the petioles to the leaf tip. Ten mature leaves of approximately equal size and maturity from each variety were used per treatment. After treatment, the leaves were kept enclosed in plastic petri dishes containing a moistened piece of filter paper on the bottom in order to keep the relative humidity high and to prevent drying of the droplets.

For the test on the rate of absorption and translocation of 2,4-D by leaves of the 'Pineapple' and 'Valencia' orange varieties, treated leaves were removed at time intervals of 2, 4, 6, 8, 12, 18 and 24 hours. At each time of sampling, the treated leaves were removed from the moist chamber and a disc 5 mm in diameter was cut from the leaf so that the center of the leaf disc was where the drop was placed. After the leaf disc was removed, the remaining leaf was frozen immediately and freeze-dried, using a Virtis Model No. 10-145 ML freeze-dryer. To keep the leaves from curling during freeze-drying, the leaves were held flat by placing them between 2 pieces of filter paper placed between a 10 x 15 cm piece of 1/2-inch mesh hardware cloth, folded in half with the open ends held together with clips. The leaves, mounted in the simple presses, were placed in small vacuum chambers, following the technique used by Talton (84).

After drying, the leaves were mounted on cardboard and covered with a 1 mill plastic mylar film. The mounted leaves were then placed next to no screen, Royal Blue, medical X-ray film to obtain autoradiograms of the leaves. After the exposure for 10-15 days, the mounts were removed and the films developed to obtain autoradiograms.

Absorption Through the Leaf Surface Using Agar Blocks

Small agar discs were prepared by placing 1 ml of 2% liquified agar, plus 1 ml of 2,4-D-1-^{14}C, into a small glass tubing of 5 mm I.D., extruding the agar after solidification and cutting it into discs 2 mm thick. Each agar disc contained 2,4-D-1-^{14}C yielding 225,000 ± 35,000 cpm as determined by scintillation techniques.

One agar disc was placed on the upper leaf surface just to the side of the mid-vein approximately one-half the distance from the petiole to the tip. For each treatment, 15 leaves of each variety were used. Only mature leaves of equal size were chosen for the test. For comparing the absorption rate of 2,4-D by leaves of 'Pineapple' and 'Valencia' orange varieties, agar discs and treated leaves were removed and placed inside counting vials to determine radioactivity by scintillation techniques using Bray's (15) mixture for aqueous solutions.

Of the 15 leaves, 5 were selected and 5 mm discs of leaf blades were removed as close as possible to the petiole. The discs were placed in counting vials to determine radioactivity. The rest of the leaves were used to prepare autoradiograms, using the technique of freeze-drying and film exposure described previously.

Uptake of 2,4-D-^{14}C Through Petioles

Eight leaves of 'Pineapple' and 'Valencia' oranges were cut under water at the petiole-stem union, leaf blade blotted dry and the cut end of the petioles placed in a 5 ml beaker containing a mixture of 2 ml of unlabeled 2,4-D plus 200 μl of radioactive 2,4-D-^{14}C (2,4-D-1-^{14}C or 2,4-D-2-^{14}C), yielding 1,000,000 ± 100,000 cpm. Both beaker and leaves were enclosed in a 750 ml, darkened chamber. For comparing rates of absorption of 2,4-D by leaves of both varieties, treated leaves were removed at 6, 12, 24 and 48 hours. At each time of sampling 2 leaves were removed. One of the leaves was freeze-dried and an autoradiogram prepared. The other leaf was ground in a Serval Omni-mixer (16,000 rpm for 3 minutes) with 80% ethanol. After steeping for 4-5 minutes, the mixture was filtered and the radioactivity in the solution determined by scintillation.

Decarboxylation Studies

Attached Leaves

Decarboxylation studies on intact leaves were made using techniques described by Talton (84). Briefly, an intact leaf was enclosed in a micro-leaf chamber constructed of plexiglass. The volume of the microchamber was approximately 3 times that of the leaf. The microchamber was in a flow system as follows: using a small diaphragm pump, air was pulled through a train of 10% NaOH solution to remove the CO_2, a H_2O scrubbing tower, a manifold with several outlets to 3 microchambers and then through 1 ml of 20% KOH to remove respired CO_2. Each chamber had a trapping solution and was monitored by scintillation techniques to determine $^{14}CO_2$ evolved from the leaves.

Three leaves of equal size and age were chosen and treated with 10 1 of 2,4-D-^{14}C. Each was placed in 1 of the 3 microchambers. The $^{14}CO_2$ evolved was sampled at 2-hour intervals for 30 hours by periodically removing 50μl of the trapping solution and determining the radioactivity. Care was taken to keep the trapping solution at a constant volume.

Detached Leaves

Branches of both 'Pineapple' and 'Valencia' oranges containing healthy, mature leaves were brought to the laboratory. Mature, uniform leaves were selected for the test, removed from the branches, washed very carefully, and the petioles were recut under water.

Four groups of 5 leaves from each of the 2 varieties were blotted and the cut ends of the petioles placed in a solution of 2 ml of unlabeled 2,4-D, plus 100μl of radioactive 2,4-D-1-^{14}C or 2,4-D-2-^{14}C. The vials containing the solution and leaves were placed inside a 750 ml darkened chamber. A counting vial containing 0.1 ml of 10% KOH was affixed with modeling clay to the inner wall near the top of the chamber and the system sealed. After 6-, 12-, 24-, and 48-hour intervals the counting vials from each group were analyzed for ^{14}C activity, using Bray's mixture and liquid scintillation counting.

Determination of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C Metabolites

In order to determine the fate of 2,4-D after it had entered the leaves through the petioles, 5 mature leaves of both 'Pineapple' and 'Valencia' orange varieties were allowed to take in 2,4-D labelled on the carboxyl or on the methyl group. The leaves were in an uptake situation for 12 hours inside a dark chamber. The $^{14}CO_2$ evolved in that period was determined by using 20% KOH and liquid scintillation detection. Upon removing the leaves from the chambers, the petioles were washed with 20 ml of 80% ethanol. The remaining solution plus the rinse was monitored for radioactivity. The leaves were ground with 50 ml of 80% ethanol and then fractionated as outlined in Fig. 1. Briefly, the ground leaves were filtered and the residue air-dried in the laboratory for 24 hours. Then 0.25 gm were taken and placed in a counting vial and analyzed for ^{14}C activity. From the ethanol extract a 25 μl aliquot was taken and the radioactivity determined. The remaining ethanolic extract was taken to dryness using a freeze-dryer. The residue left after freeze-drying was washed 3 times with a mixture of 25 ml of hexane and 25 ml of acetonitrile, and the hexane and acetonitrile phases were separated and aliquots (25 μl) removed from each for radioactivity determinations. The remaining acetonitrile fraction was concentrated to dryness using the freeze-dryer.

The residue was subjected to extraction with a mixture of water and ether (ether: H_2O, 1:1, v/v) to which 3 ml of saturate $NaHCO_3$ was added. The final solution was pH 8-10. After the ether and water fractions were separated, an aliquot of the ether fraction (25 μl) was taken for a determination of ^{14}C activity.

The aqueous fraction was adjusted to pH 2.0 with 1N H_2SO_4 and partitioned against hydrated ether. After separation, a 25 μl aliquot of the water-ether fraction was taken for radioactivity determinations.

All fractions of the extracts were reduced in volume to approximately 0.25 ml and fractionated by ascending paper chromatography, by thin-layer chromatography (TLC), and by paper electrophoresis.

Paper Chromatography

Whatman No. 1 chromatographic paper, 3 x 30 cm in size, was used for ascending chromatography of 500 μl of the different extracts. The

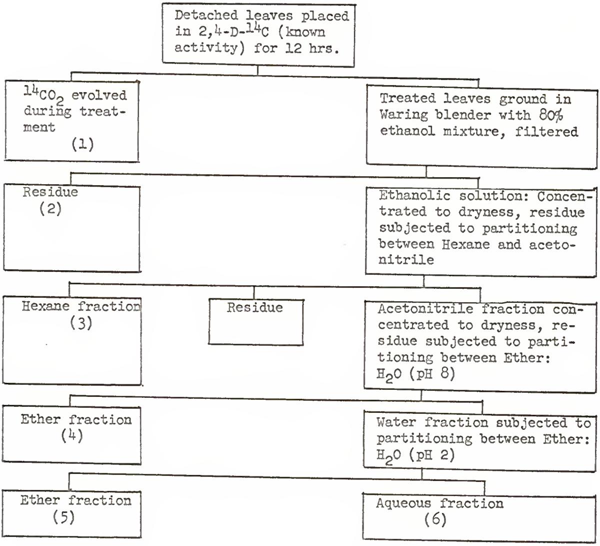

Detached leaves placed in 2,4-D-^{14}C (known activity) for 12 hrs.
$^{14}CO_2$ evolved during treatment (1)
Treated leaves ground in Waring blender with 80% ethanol mixture, filtered
Residue (2)
Ethanolic solution: Concentrated to dryness, residue subjected to partitioning between Hexane and acetonitrile
Hexane fraction (3)
Residue
Acetonitrile fraction concentrated to dryness, residue subjected to partitioning between Ether: H_2O (pH 8)
Ether fraction (4)
Water fraction subjected to partitioning between Ether: H_2O (pH 2)
Ether fraction (5)
Aqueous fraction (6)

Fig. 1.- Diagram of the fractionational procedure for 2,4-D-^{14}C metabolites from treated leaves. Numbers in parentheses indicate fractions.

solvent was very similar to the one used by Zenk (94), Isopropanol: ammonia: water (8:1:1, v/v/v) with a development time of 6-8 hours.

The paper chromatograms were scanned for radioactivity using a G-M counter. After scanning, the paper chromatograms were attached to a cardboard backing and placed inside an X-ray film to obtain autoradiograms. After 2-3 weeks, X-ray films were developed and the R_f's of the different areas of activity measured.

Thin Layer Chromatography (TLC)

Eastman thin-layer chromatogram sheets (Type K-301 R2) were used to fractionate the different extracts using a solvent system consisting of Isopropanol: ammonia: water (8:1:1, v/v/v), with a development time 3-4 hours. After drying, the TLC plates were placed inside an X-ray folder next to X-ray film, to obtain autoradiograms. After exposure for 2-3 weeks, the X-ray films were developed and the R_f's of the different radioactivity areas determined. The radioactive areas of the TLC plates of the aqueous extracts were eluted with 80% ethanol and the solution filtered. The filtrate was divided in 2 portions; one was subjected to 1N HCl and the other kept at a neutral pH. The 2 portions were reduced to dryness and then redissolved in 0.5 ml of ethanol, and again subjected to TLC.

Electrophoresis

The apparatus used was a Beckman Spinco Duostat electrophoretic system. Paper electrophoretograms were obtained,using strips, Spinco B-2 Buffer solution, and developed at 500 V for 3 hours.

After drying the paper electrophoretograms, they were scanned for radioactivity. Also, autoradiograms were prepared by exposing the paper electrophoretograms to X-ray films.

Determination of Radioactivity

Liquid Scintillation Technique

For the determination of ^{14}C present in radioactive solutions, samples of the solution were taken, usually 25 μl, and placed in counting vials together with Bray's mixture (15) for aqueous solutions (60 g naphthalene, 4 g PPO, 200 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (Scintillation grade (POPOP), 100 ml methanol, 20 ml ethylene glycol and p-dioxane to make one L). The determination of radioactivity was done using a Packard Tri-Carb Liquid Scintillation Spectrometer Series 314 E with a model 500 D automatic control.

Monitoring the Strips for Radioactivity

The radioactivity present on paper chromatograms was determined using a Geiger-Muller gas flow counter (Nuclear Chicago Model 1620 B) equipped with a strip scanner (Nuclear Chicago Model C-100A) attached to a 1 m A recorder. This produced graphs of the radioactive distribution on the paper chromatograms.

Preparations of Autoradiograms

Autoradiograms of leaves, paper chromatograms and electrophoretograms were prepared by attaching them to a cardboard and then covering them with a piece of 1 mill mylar film to prevent chemical reactions with the film emulsion. The mounts were placed next to no-screen, Royal-Blue, medical X-ray film (RB-54), inside an X-ray film holder and pressed firmly with weight to permit good contact between leaves or paper strips and the film. TLC sheets were prepared in the same way, but they were placed next to the X-ray film without covering them with the mylar film. After 2-3 weeks, X-ray films were developed following the usual procedures.

The Effect of 2,4-D on Pathways of Glucose, Manometric System

The procedure for this study was almost identical to that used by Humphreys and Dugger (49). Glucose labeled with carbon-14 in the C-1 and C-6 position (Glucose-1-^{14}C and Glucose-6-^{14}C) was used. They were obtained from New England Nuclear Corp., Boston, Mass. For the Glucose-1-^{14}C, 1.8 mg contained 0.1 mc of ^{14}C (specific activity, 10 mc/m mole), and for the glucose-6-^{14}C, 4.7 mg contained 0.1 mc of ^{14}C (specific activity, 3.83 mc/m mole). Solutions used for the test contained the labeled glucose plus enough unlabeled glucose to make an aqueous solution of 80.0 mgs/10 ml (specific activity, 0.4 mc/m mole).

Leaves of both varieties, 'Pineapple' and 'Valencia' orange, were washed with distilled water, blotted, and the cut end of the petiole placed in vials containing either 10^{-2} M solution of potassium phosphate buffer, pH 5.3, or buffer plus 10^{-3} M 2,4-D. The vials and leaves were then placed in the dark for 12 hours at room temperature. After incubation the leaves were removed and leaf discs (5 mm in diameter) were removed. A sample of leaf discs of 0.25 gms were used per treatment.

The manometric experiments were conducted using a Warburg apparatus built by the Precision Scientific Co. Ser. No. M-8. In all experiments, 8 equal samples of leaves discs were placed in standard 10 ml conical

Warburg vessels, having a side arm. The vessels contained 1.5 ml of 10^{-2} M potassium phosphate buffer, pH 5.3, in the main compartment; 0.1 ml of 20% KOH in the center well wicked in a folded 1 cm^2 filter paper piece; and 0.2 ml of 10 N H_2SO_4 in the side arm. Eight vessels, containing leaf discs from 2,4-D treated leaves and 8 vessels containing leaf discs treated with just buffer were used. Four of the 8 in each group received 0.05 ml of Glucose-1-^{14}C; the other 4 vessels in each group received 0.05 ml of Glucose-6-^{14}C. The added radioactive glucose had an activity of approximately 600,000 cpm as measured under the conditions of these tests. Immediately after adding the glucose, the vessels were attached to the manometers and placed in the water bath at 30 ± 0.05° C. Air was the gas phase in the vessels.

Readings of the manometers for oxygen uptake were made every 15 minutes and the vessels were shaken in the dark for 3.5 hours. At the end of this period, the acid was tipped from the side arm into the main compartment to stop the reactions. The shaking of the vessels was continued for an additional 15 minutes. Then the filter paper plus KOH was removed from the center well and placed in counting vials for determination of $^{14}CO_2$,using liquid scintillation techniques.

Closed Non-manometric System Used to Study the Effect of 2,4-D on Glucose Metabolism

Mature leaves of both varieties, 'Pineapple' and 'Valencia' orange, were brought to the laboratory, washed very carefully, petioles recut under water, leaf blade blotted and weighed to obtain 5.0 gm samples. Four groups of 5 leaves for each variety were used. Half of the samples were placed in vials containing radioactive glucose and buffer, and the other half in solutions of radioactive glucose, plus buffer and 2,4-D. One-half of the vials received G-1-^{14}C and the other half G-6-^{14}C, yielding around 12,000,000 cpm.

The vials containing the explants and the radioactive solutions were placed in 750 ml amber chambers. A counting vial containing 5 ml of 20% KOH was affixed inside the chambers as described before to trap the evolved $^{14}CO_2$, and the whole system was sealed air tight.

To sample the 20% KOH solution for radioactivity, a hypodermic needle was passed through a septum mounted in the wall of the chamber to below the surface of the KOH solution. Periodically, 0.2 ml sample would be removed by syringe, followed by readjusting the volume of KOH in the chamber to 5 ml.

RESULTS

Tests were conducted using 2,4-D-1-^{14}C (10^{-3} M) to determine the extent and pattern of distribution of radioactive materials in the leaf from drops placed on the surface of the leaf. Care was taken to keep the droplets from evaporating by placing the leaves, still attached to the plant, in plastic petri dishes. In Figs. 2 through 5, autoradiograms of leaves treated on the adaxial side are presented, and Fig. 6 represents autoradiograms of leaves treated on the abaxial side. The results indicated that either 2,4-D-1-^{14}C was not taken into the leaf or once it entered the leaf, either could not be detected because of the rather thick leaf which absorbed the weak Beta particles emitted by ^{14}C decay, or the 2,4-D-1-^{14}C was metabolized quickly with the subsequent loss of the ^{14}C. Comparisons of the autoradiograms of leaves did not give an indication of the extent or pattern of distribution of 2,4-D-1-^{14}C in orange leaves but did reveal several points. First, there was radioactivity in the leaf since the injured edge of leaf around the spot where the leaf disc was removed was nearly always indicated on the autoradiograms. Secondly, the techniques detected radioactivity on the surface of the leaf (note the indication of radioactivity on surface of the leaf in Fig. 6). When the 2,4-D-1-^{14}C was applied to the lower surface of the leaf, it was difficult to remove quantitatively by washing after the designated time of uptake. This surface contamination was clearly evident. Thirdly, there was no apparent difference between the autoradiograms of 'Pineapple' and 'Valencia' leaves.

Fig. 2.- Autoradiographs of 'Pineapple' and 'Valencia' orange leaves 2, 4, and 8 hours after treatment with a 10 μl drop of 2,4-D-1-^{14}C (730,000 ± 30,000 cpm) on the adaxial side at the point of the arrow. After the designated times, a 5 mm leaf disc was removed prior to drying the leaf.

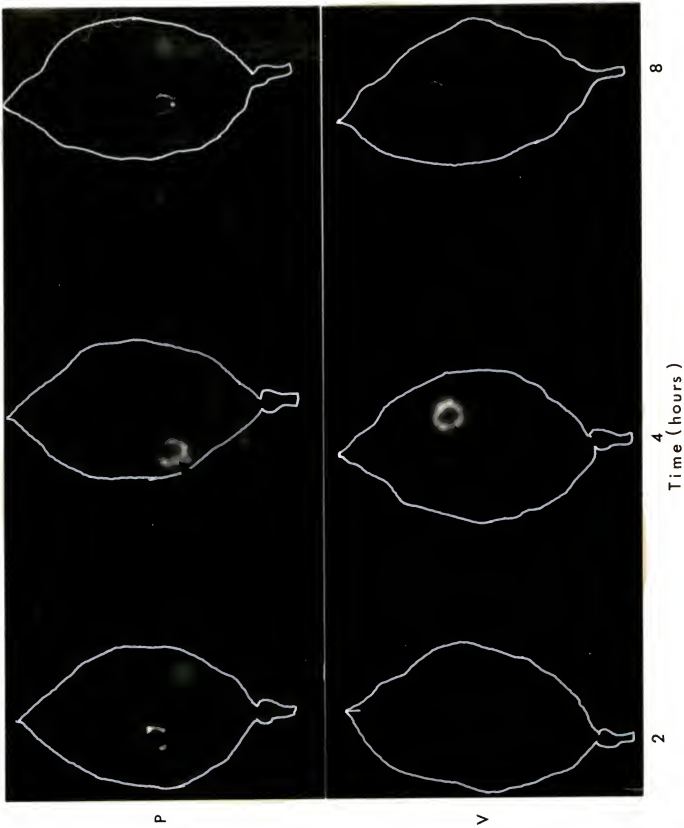

P
V
2
4
8
Time (hours)

Fig. 3.- Autoradiographs of 'Pineapple' and 'Valencia' orange leaves 12, 18 and 24 hours after treatment with a 10 µl drop of 2,4-D-1-^{14}C (730,000 ± 30,000 cpm) on the adaxial side at the point of the arrow. After the designated times a 5 mm leaf disc was removed prior to drying the leaf.

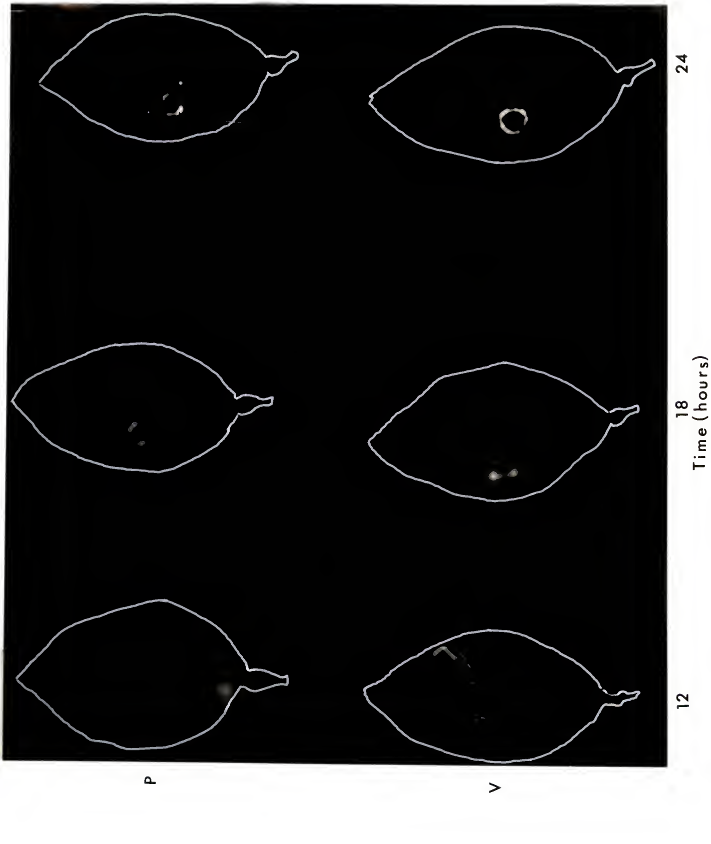

P
V
12
18
24
Time (hours)

Fig. 4.- Autoradiograms of 'Pineapple' (A, B) and 'Valencia' (C, D) orange leaves 24 (A, C) and 48 (B, D) hours after treatment with a 10 µl drop of 2,4-D-1-^{14}C (730,000 ± 30,000 cpm) on the adaxial side at the point of the arrow. After the designated times, a 5 mm leaf disc was removed prior to drying the leaf.

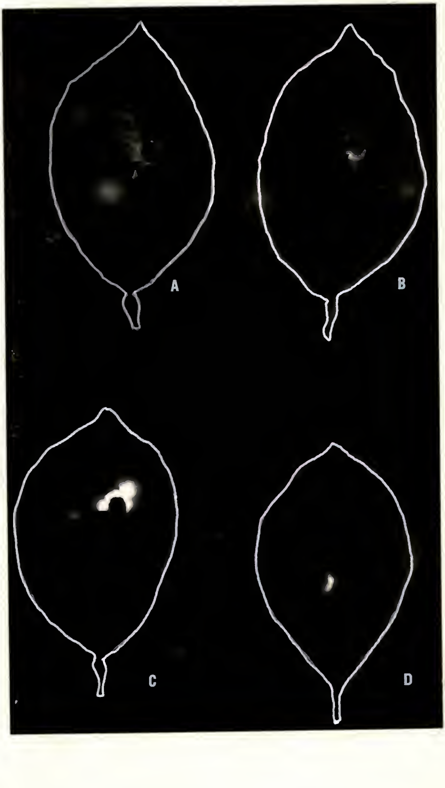

A
B
C
D

Fig. 5.- Autoradiograms of 'Pineapple' (A, B) and 'Valencia' (C, D) orange leaves 72 (A, C) and 96 (B, D) hours after treatment with a 10μl drop of 2,4-D-1-^{14}C (730,000 ± 30,000 cpm) on the adaxial side at the point of the arrow. After the designated times, a 5 mm leaf disc was removed prior to drying the leaf.

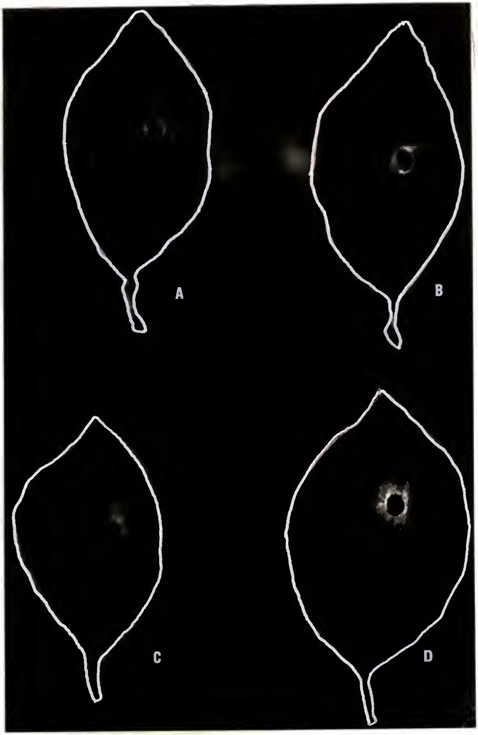
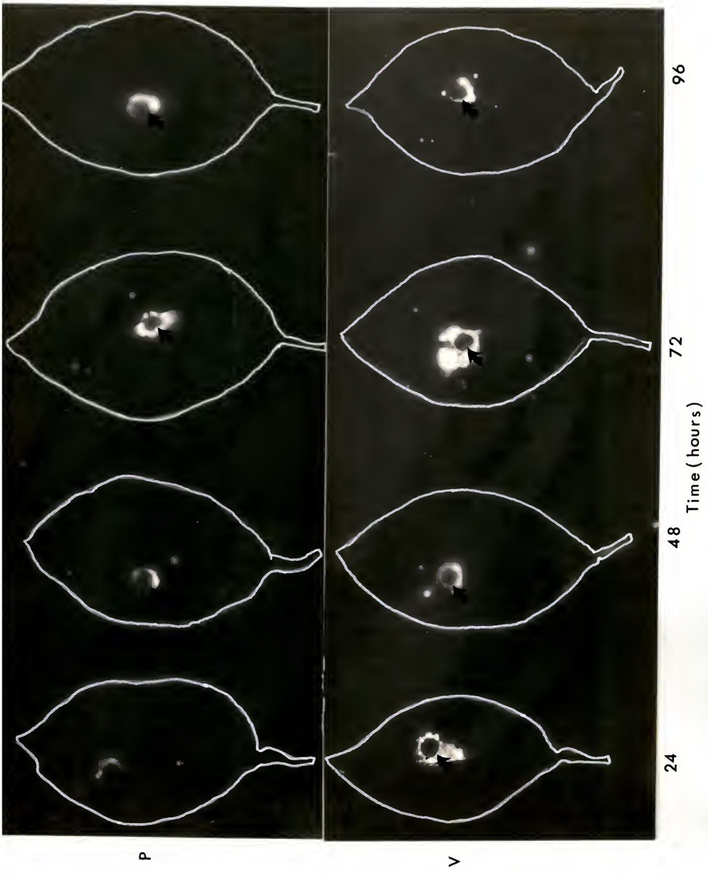

A
B
C
D

Fig. 6.- Autoradiograms of 'Pineapple' and 'Valencia' orange leaves 2, 4, 48, 72 and 96 hours after treatment with a 10 µl drop of 2,4-D-1-^{14}C (730,000 ± 30,000 cpm) on the adaxial side at the point of the arrow. After the designated times, a 5 mm leaf disc was removed prior to drying the leaf.


P
V
24
48
72
96
Time (hours)

To determine if the 2,4-D-1-^{14}C was not getting into the leaf (due to the possible drying of the drops on the leaf surface), the radioactive compound was incorporated in an agar disc (2 mm x 6 mm) and the agar disc placed on the adaxial surface of the leaf. There was very little activity indicated on the autoradiograms except beneath the point of application of the agar block (Fig. 7). The small white dots in the whitish circle, indicate small areas of very intense radioactivity. The distribution of the small white dots per unit of area compares reasonably well with number of oil glands on the adaxial surface of the leaf (approximately 2 mm^2).

In addition to determining the amount of uptake of 2,4-D-1-^{14}C from agar discs by autoradiography, a leaf disc 5 mm in diameter was removed from the leaf adjacent to the abscission zone. This disc included the mid-rib and leaf lamina and was assayed for radioactivity, using scintillation counting techniques. Very little if any radioactivity had been translocated to this area in 4 hours (Table 1). It was interesting to note that the place where the leaf disc was removed from the leaf was not evident on the autoradiograms of these same leaves (Fig. 7).

To determine if and to what extend the 2,4-D-1-^{14}C was getting into the leaf and the loss via $^{14}CO_2$, the solution of 2,4-D-1-^{14}C was applied to the leaf surface as 2 drops of 10 μl each. From leaves chosen for uniformity, samples were taken at 2, 4, 6, 12, 18 and 24 hours to determine the amount of ^{14}C appearing as CO_2, the amount of radioactivity in the leaf disc just underneath the point of application and the amount of radioactivity in the rest of the leaf extractable with 80% ethanol. As shown in Tables 2 and 3, the radioactivity of the leaf with time

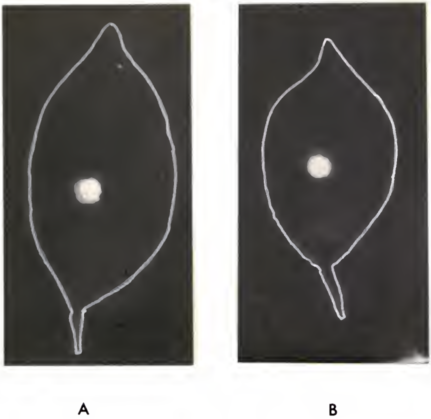


Fig. 7.- Autoradiogram of 'Pineapple' (A) and 'Valencia' (B) orange leaves after treatment with 2,4-D-1-^{14}C using agar discs. The period of uptake was for 4 hours from agar discs containing 5×10^{-4} M of 2,4-D-1-^{14}C (227,445 ± 35,000 cpm).

Table 1.- Radioactivity (cpm) appearing after 1, 2, and 4 hours near the abscission zone after uptake through leaf surface from 2,4-D-1-^{14}C incorporated in agar discs.[a]

	Time		
Variety	1 hr.	2 hrs.	4 hrs.
Pineapple	-[b]	2	2
Valencia	-	-	12

[a] Activity of the agar discs before placing them on top of the leaves $\bar{x}$ 227,445 ± 35,000 cpm.

[b] Average of 10 replications.

was neither significantly different in the leaf disc nor in the 80% ethanol extractable portion. However, the most striking finding was that radioactive extracts could be obtained from the leaves after 2 hours was not significantly different from other sampling periods. This indicated a relatively large quantity of 2,4-D had entered the leaf in a short period of time. The reason for the lack of additional uptake after 2 hours could not be due to the drying of the droplets on the leaf surface.

The increased $^{14}CO_2$ production was significantly different at each period of sampling, but it was apparent that only a small fraction of the 2,4-D-1-^{14}C taken into the leaf was yielding $^{14}CO_2$.

Uptake of 2,4-D-1-^{14}C Through Petioles of Detached Leaves

When leaves of 'Pineapple' and 'Valencia' orange were placed in solution of 2,4-D-1-^{14}C or 2,4-D-2-^{14}C, more 2,4-D absorbed by the leaf, but autoradiography of the leaves yielded poor patterns and the extent of uptake could not be ascertained with any degree of reliability (Figs. 8 and 9). More radioactivity was present in both 'Pineapple' and 'Valencia' leaves after 24- and 48-hour period than after 6 and 12 hours of uptake, but there was no discernible difference between the 2 varieties. In addition to following the uptake of 2,4-D-1-^{14}C through the petioles, 2,4-D-2-^{14}C was also used. The identical conditions of poor patterns of distribution and evidence of little radioactivity getting to the films were obtained.

As with the surface application test, the uptake of 2,4-D-1-^{14}C was determined by extracting the tissue with 80% ethanol and determining

Table 2.- Uptake of 2,4-D-1-^{14}C through the surface of 'Pineapple' orange leaves and the subsequent $^{14}CO_2$ evolution.[a]

Time	Activity of an 80% ethanol extract of the leaf[b] (cpm)	Activity of the leaf disc[c] (cpm)	$^{14}CO_2$ evolution (cpm)
0-2 hrs.	119,866	11,500	2
0-4 hrs.	125,250	12,000	26
0-6 hrs.	104,099	17,350	47
0-12 hrs.	82,658	15,650	126
0-18 hrs.	157,462	15,050	204
0-24 hrs.	104,037	13,650	283

[a] The 2,4-D-1-^{14}C was applied to the surface of the leaf as one 10 μl drops on one side of the mid-rib.

[b] Leaf minus the leaf disc.

[c] Leaf disc from section of leaf in direct contact with 2,4-D-1-^{14}C solution which was surface washed prior to counting.

Table 3.- Uptake of 2,4-D-1-^{14}C through the surface of 'Valencia' orange leaves and the subsequent $^{14}CO_2$ evolution.[a]

Time	Activity of an 80% ethanol extract of the leaf[b] (cpm)	Activity of the leaf disc[c] (cpm)	$^{14}CO_2$ evolution (cpm)
0-2 hrs.	113,720	13,850	0
0-4 hrs.	114,952	12,350	19
0-6 hrs.	121,670	14,500	42
0-12 hrs.	147,675	18,000	114
0-18 hrs.	113,972	15,300	191
0-24 hrs.	102,631	17,000	259

[a] The 2,4-D-1-^{14}C was applied to the surface of the leaf as one 10 μl drops on one side of the mid-rib.

[b] Leaf minus leaf disc.

[c] Leaf disc from section on leaf in direct contact with 2,4-D-1-^{14}C solution which was surface washed prior to counting.

Fig. 8.- Autoradiograms of 'Pineapple' leaves 6 (A), 12 (B), 24 (C) and 48 (D) hours after treatment with 2,4-D-1-^{14}C (10^{-3} M). The uptake was through the petiole, and 2 ml of 2,4-D solution contained specific activity of 0.1 mc/m mole.

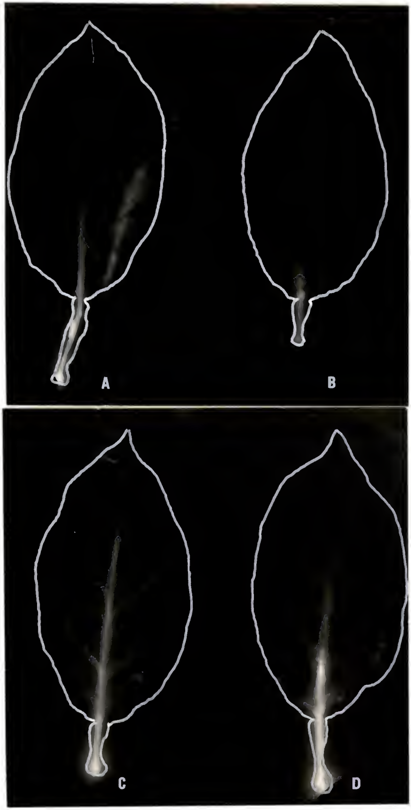
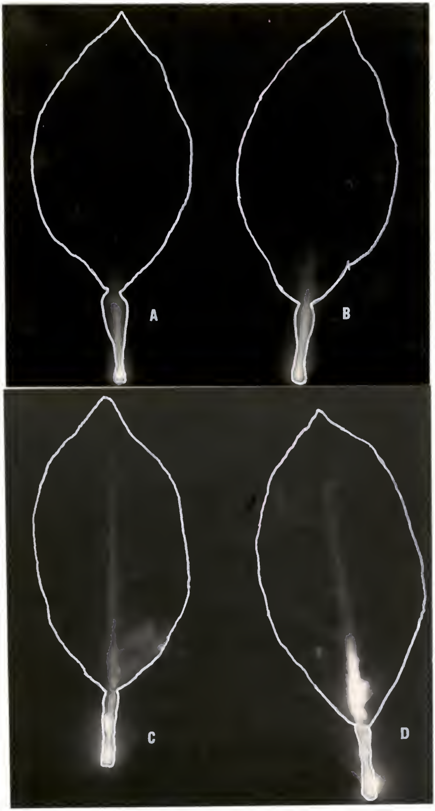

A
B
C
D

Fig. 9.- Autoradiogram of 'Valencia' orange leaves 6 (A), 12 (B), 24 (C) and 48 (D) hours after treatment with 2,4-D-1-^{14}C (10^{-3} M). The uptake was through the petiole, and 2 ml of 2,4-D solution contained specific activity of 0.1 mc/m mole.


A
B
C
D

the radioactivity by using scintillation techniques.

In this test, the leaves were placed in 2,4-D-1-^{14}C solution for 24 hours and the $^{14}CO_2$ evolved during the uptake period was trapped and counted. As shown in Table 4, there was a definite uptake and metabolism of a portion of 2,4-D-1-^{14}C by both varieties. When compared to the uptake by surface application, the petiole uptake of 2,4-D-1-^{14}C was much greater, and was the method used to get 2,4-D into the tissue for the subsequent tests.

Patterns of the Evolution of $^{14}CO_2$ from Tissue Treated with 2,4-D-1-^{14}C and 2,4-D-2-^{14}C

There was $^{14}CO_2$ evolved from the orange tissues when treated with 2,4-D-1-^{14}C as shown by surface application tests on intact leaves (Table 2 and 3) and by the petiole-uptake test when 'Pineapple' and 'Valencia' leaves were treated with both 2,4-D-1-^{14}C and 2,4-D-2-^{14}C.

To obtain an accurate pattern of the evolution of $^{14}CO_2$ from the metabolism of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C by both 'Pineapple' and 'Valencia' leaves, detached leaves were treated with the carbon-labeled compound and $^{14}CO_2$ trapped and radioactivity determined. As shown by the counts obtained (Table 5) and a graphic presentation of the results (Fig. 12) there was almost a linear increase in $^{14}CO_2$ production with time evolving from both varieties with both 2,4-D-1-^{14}C and 2,4-D-2-^{14}C. There was no difference between the 2 varieties in the pattern of $^{14}CO_2$ production. However, there was a difference in the amount of $^{14}CO_2$ evolving from tissues treated with 2,4-D-1-^{14}C as compared to tissues treated with 2,4-D-2-^{14}C. There was approximately 4 times as much $^{14}CO_2$ produced from 2,4-D-1-^{14}C as from 2,4-D-2-^{14}C.

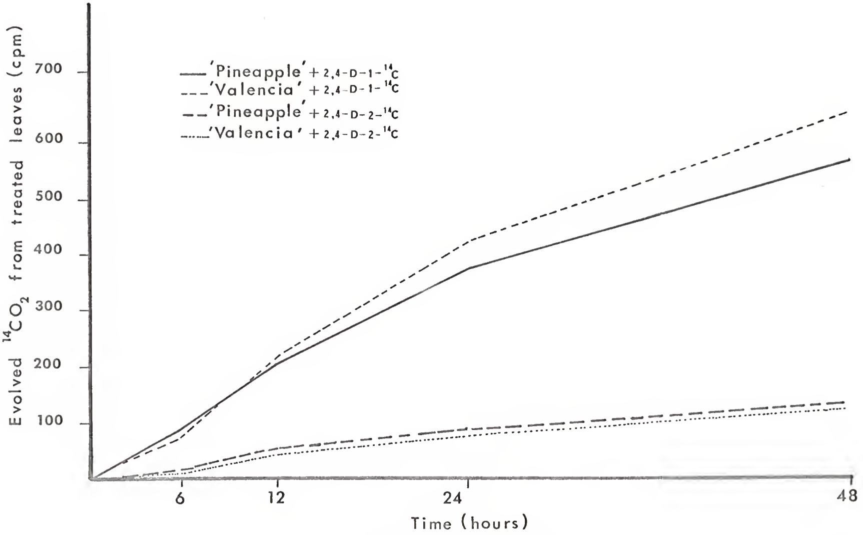


Fig. 10.- Production in time of $^{14}CO_2$ from 'Pineapple' and 'Valencia' orange leaves treated with 2,4-D-1-^{14}C and 2,4-D-2-^{14}C.

Table 4.- Uptake of 2,4-D-1-^{14}C through the petioles of orange leaves

Variety	Radioactivity of the uptake solution (cpm)	Radioactivity in an 80% ethanol extract of the leaf	$^{14}CO_2$ evolved in 24 hrs. (%)[a]
Pineapple			
Exp. I	1,897,261	358,401	0.029
Exp. II	1,153,195	399,019	0.035
Exp. III	3,673,059	507,719	0.14
Valencia			
Exp. I	1,898,739	659,643	0.035
Exp. II	1,007,895	286,395	0.028
Exp. III	3,842,075	745,434	0.19

[a] Per cent of radioactivity extractable from the leaf with 80% ethanol.

Table 5.- Production of $^{14}CO_2$ (cpm) from either 'Pineapple' or 'Valencia' orange leaves treated with either 2,4-D-1-^{14}C or 2,4-D-2-^{14}C.[a]

		cpm			
Variety	Treatment	6 hrs.	12 hrs.	24 hrs.	48 hrs.
Pineapple	2,4-D-1-^{14}C	91	208	373	561
Valencia	2,4-D-1-^{14}C	75	224	428	648
Pineapple	2,4-D-2-^{14}C	18	53	88	135
Valencia	2,4-D-2-^{14}C	15	49	86	128

[a] Radioactivity of the solutions used for treatment was 450,000 ± 26,000 cpm.

Fate of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C in Leaves

A solvent fractionational procedure was used to partially separate the metabolites from an 80% ethanolic extract from tissues treated with 2,4-D-1-^{14}C and 2,4-D-2-^{14}C. Table 6 represents the pattern of distribution and the % recovery of pure 2,4-D-1-^{14}C in the fractionational procedure outline in Fig. 1. Under the conditions of this fractionation, a large portion of the radioactivity remains in the aqueous fraction.

When pure 2,4-D-1-^{14}C was added to an 80% ethanolic extract from either 'Pineapple' or 'Valencia' orange tissue, the pattern of distribution of radioactivity in the various fractions and the % recovery are as shown in Tables 7 and 8. There was a considerable change in the radioactivity appearing in the various fractions after the addition of just the ethanolic-soluble components to the pure 2,4-D. It was noted that the soluble materials from 'Pineapple' leaves caused a different pattern of distribution from that of materials from 'Valencia' leaves. Therefore, the distribution patterns of 2,4-D-1-^{14}C with the ethanolic-soluble components from each tissue was used as the control for that tissue.

When 2,4-D-1-^{14}C or 2,4-D-2-^{14}C was applied to either 'Pineapple' or 'Valencia' leaves by petiole-uptake 12 hours prior to extracting the tissue with 80% ethanol and subjecting the extract to the solvent partitioning procedure, the patterns of distribution of the radioactivity in the various fractions are as shown in Tables 9, through 12. The pattern of distribution of radioactivity in the various factions were quite different when living tissues were introduced into the system (Table 13).

With 'Pineapple' leaves, the tissue reactions caused a greater

Table 6.- Fractionation of pure 2,4-D-1-^{14}C by the solvent partitioning procedure.[a]

Fractions	Radioactivity[b] (cpm)	% Recovery
3- Hexane soluble	81,640	7.49
4- Ether soluble, pH 8	51,548	4.7
5- Ether soluble, pH 2	51,568	4.72
6- Aqueous	1,002,562	92.07
7- Residue	11,040	1.01
Total Recovery		115.0

[a] See Fig. 1 for diagram of fractionation procedure.

[b] Average of 2 replications.

Table 7.- Fractionation of 2,4-D-1-^{14}C when added to the ethanolic-soluble components from 'Pineapple' orange leaves.[a]

Fractions[b]	Radioactivity[c] (cpm)	% Recovery
2- Residue	2,062	0.24
3- Hexane soluble	158,324	18.39
4- Ether soluble, pH 8	59,150	6.87
5- Ether soluble, pH 2	63,200	7.33
6- Aqueous	448,872	52.13
7- Residue	13,866	1.61
Total Recovery		86.57

[a] 2,4-D-1-^{14}C added to the tissue immediately after grinding in 80% ethanol.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 2 replications.

Table 8.- Fractionation of 2,4-D-1-^{14}C when added to the ethanolic-soluble compounds from 'Valencia' orange leaves.[a]

Fractions[b]	Radioactivity[c] (cpm)	% Recovery
2- Residue	764	0.08
3- Hexane-soluble	64,516	6.53
4- Ether soluble, pH 8	86,380	10.36
5- Ether soluble, pH 2	279,268	28.25
6- Aqueous	387,750	39.23
7- Residue	14,670	1.48
Total Recovery		84.30

[a] 2,4-D-1-^{14}C added to the tissue immediately after grinding in 80% ethanol.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 2 replications.

Table 9.- Radioactivity in the various solvent partition fractions from an 80% ethanolic extract from 'Pineapple' orange leaves treated with 2,4-D-1-^{14}C.[a]

Fractions[b]	Radioactivity[c] (cpm)	% Recovery
1- $^{14}CO_2$	277	0.04
2- Residue	18,553	2.66
3- Hexane-soluble	148,181	23.76
4- Ether soluble, pH 8	87,910	15.24
5- Ether soluble, pH 2	66,874	11.04
6- Aqueous	180,568	30.83
Residue	86,839	10.28
Total Recovery		93.85

[a] 2,4-D-1-^{14}C applied to leaves by petiole uptake 24 hours prior to extraction.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 3 replications.

Table 10.- Radioactivity in the various solvent partition fractions from an 80% ethanolic extract from 'Valencia' orange leaves treated with 2,4-D-1-^{14}C.[a]

Fractions[b]	Radioactivity[c] (cpm)	% Recovery
1- $^{14}CO_2$	412	0.05
2- Residue	9,708	1.18
3- Hexane soluble	160,702	22.67
4- Ether soluble pH 8	83,045	12.39
5- Ether soluble pH 2	80,741	12.73
6- Aqueous	176,933	32.54
Residue	86,536	10.82
Total Recovery		92.38

[a] 2,4-D-1-^{14}C applied to leaves by petiole uptake 24 hours prior to extraction.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 3 replications.

Table 11.- Radioactivity in the various solvent partition fractions from an 80% ethanolic extract from 'Pineapple' orange leaves treated with 2,4-D-2-^{14}C.[a]

Fractions[b]	Radioactivity[c] (cpm)	% Recovery
1- $^{14}CO_2$	54	0.01
2- Residue	1,560	0.29
3- Hexane soluble	124,546	23.5
4- Ether soluble pH 8	52,492	9.90
5- Ether soluble pH 2	49,686	9.37
6- Aqueous	198,770	37.50
Residue	18,156	3.42
Total Recovery		83.99

[a] 2,4-D-1-^{14}C applied to leaves by petiole uptake 24 hours prior to extraction.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 3 replications.

Table 12.- Radioactivity in the various solvent partition fractions from an 80% ethanolic extract[b] from 'Valencia' orange leaves treated with 2,4-D-2-^{14}C.[a]

Fractions	Radioactivity[c] (cpm)	% Recovery
1- $^{14}CO_2$	51	0.01
2- Residue	805	0.17
3- Hexane soluble	102,132	21.80
4- Ether soluble pH 8	53,144	11.35
5- Ether soluble pH 2	46,828	10.00
6- Aqueous	152,860	32.65
Residue	21,538	4.60
Total Recovery		80.58

[a] 2,4-D-1-^{14}C applied to leaves by petiole uptake 24 hours prior to extraction.

[b] See Fig. 1 for diagram of fractionation procedure.

[c] Average of 3 replications.

Table 13.- Comparison of the radioactivity from 2,4-D-1-^{14}C and 2,4-D-2-^{14}C in the various fractions from the fractionational procedure as influenced by living and non-living tissues.

	Radioactivity recovered from 'Pineapple' tissue (%)[a]			Radioactivity recovered from 'Valencia' tissue (%)[b]		
	Non-living	Living		Non-living	Living	
Fractions	2,4-D-1-^{14}C	2,4-D-1-^{14}C	2,4-D-2-^{14}C	2,4-D-1-^{14}C	2,4-D-1-^{14}C	2,4-D-2-^{14}C
1- $^{14}CO_2$	-	0.04	0.01	-	0.05	0.01
2- Residue	0.24	2.66	0.29	0.08	1.18	0.17
3- Hexane-soluble	18.39	23.76	23.5	6.53	22.67	21.80
4- Ether soluble, pH 8	6.87	15.24	9.90	10.36	12.39	11.35
5- Ether soluble, pH 2	7.33	11.04	9.37	28.25	12.73	10.00
6- Aqueous	52.13	30.83	37.50	39.23	32.54	32.65
7- Residue	1.61	10.28	3.42	1.48	10.82	4.60
Total Recovery	86.57	93.85	83.99	84.30	92.38	80.58

[a] See Tables 7, 9 and 11 for treatments.

[b] See Tables 8, 10 and 12 for treatments.

percentage of the radioactivity to occur in the hexane and ether fractions with a concomitant decrease in the aqueous fractions. The patterns of distributions were essentially the same for both 2,4-D-1-^{14}C and 2,4-D-2-^{14}C fractions from living tissues. With 'Valencia' leaves, the tissue reactions caused a greater percentage of the radioactivity to occur in the hexane fraction with a concomitant decrease in the ether II and aqueous fractions. Here again, the pattern of distribution for both 2,4-D-1-^{14}C and 2,4-D-2-^{14}C was essentially the same.

The difference in distribution of radioactivity in the living versus nonliving tissue systems indicates metabolism of 2,4-D did occur and that the difference between 'Pineapple' and 'Valencia' tissue may indicate that the 2 varieties metabolize 2,4-D to different products. Therefore, further purification of the radioactivity components was attempted.

Paper Chromatography of the Fractions Obtained by Solvent Partitioning

The various fractions obtained from the solvent partitioning study were subject to paper chromatography. The resulting paper chromatograms were scanned for radioactivity (Figs. 11 through 18). Each graph represents 2 fractions: the 2,4-D-1-^{14}C added to alcohol-killed tissue during the extraction and 2,4-D-1-^{14}C added to living tissue 24 hours prior to extraction.

A compilation of R_f's of the various radioactive components clearly visible on the scans of the paper chromatograms of the fractions can be found in Table 14. It can be seen from the graphs and the compilation

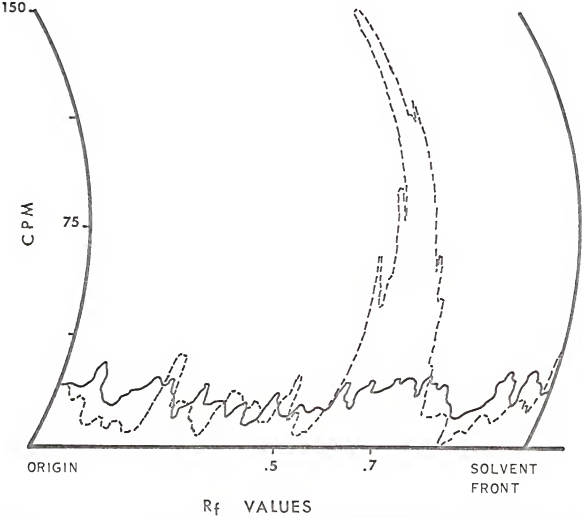


Fig. 11.- Distribution of ^{14}C activity of the Hexane fraction from 'Pineapple' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water. (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

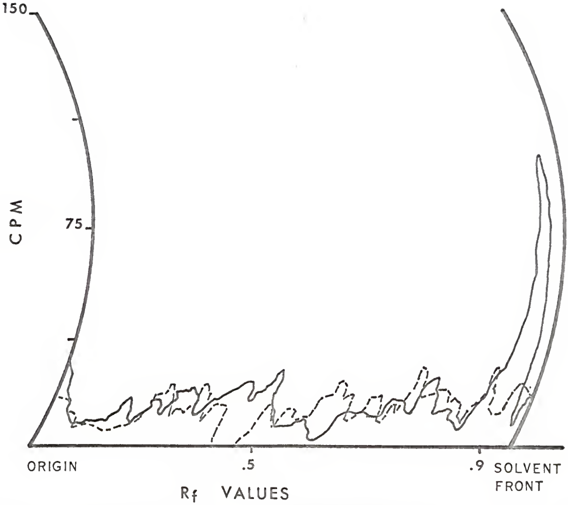


Fig. 12.- Distribution of ^{14}C activity of the Ether I fraction from 'Pineapple' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living; dotted line: 2,4-D-1-^{14}C added to extract).

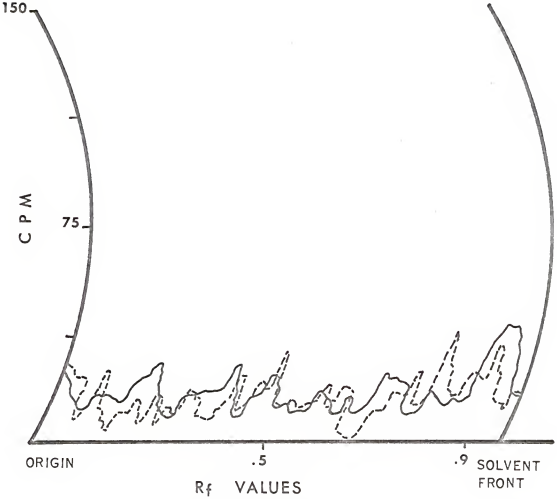


Fig. 13.- Distribution of ^{14}C activity of the Ether II fraction from 'Pineapple' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

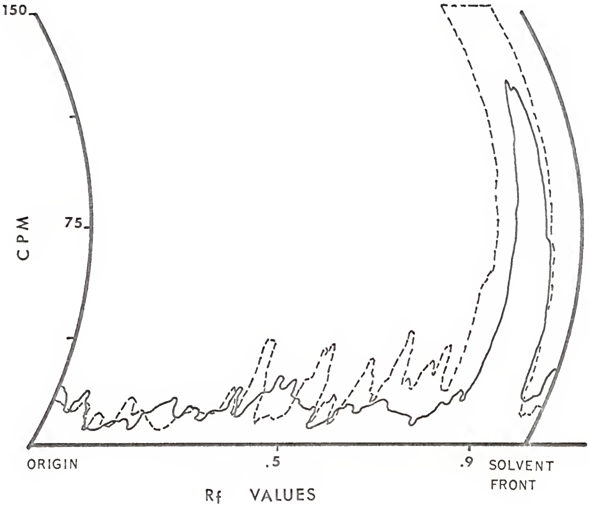


Fig. 14.- Distribution of ^{14}C activity of the Aqueous fraction from 'Pineapple' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract)

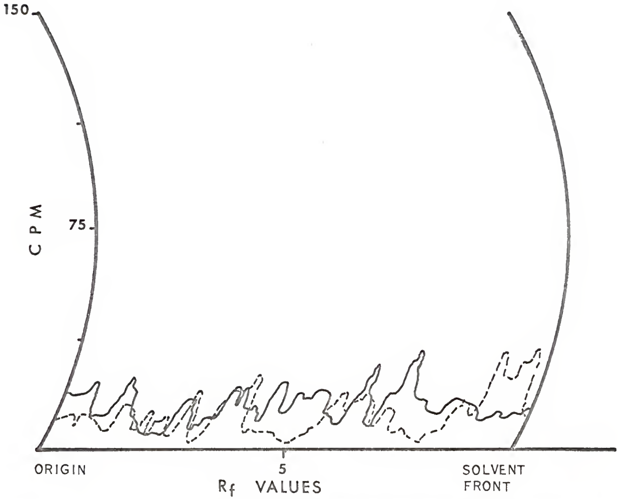


Fig. 15.- Distribution of ^{14}C activity of the Hexane fraction from 'Valencia' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

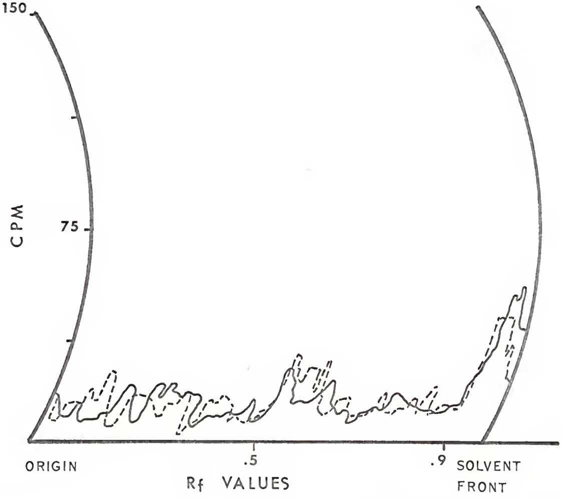


Fig. 16.- Distribution of ^{14}C activity of the Ether I fraction from 'Valencia' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

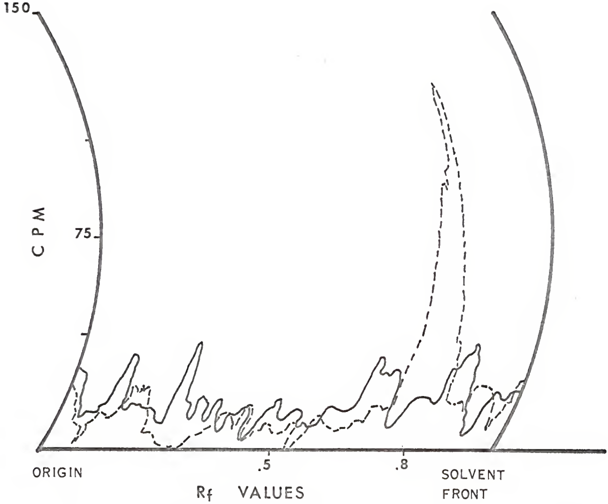


Fig. 17.- Distribution of ^{14}C activity of the Ether II fraction from 'Valencia' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

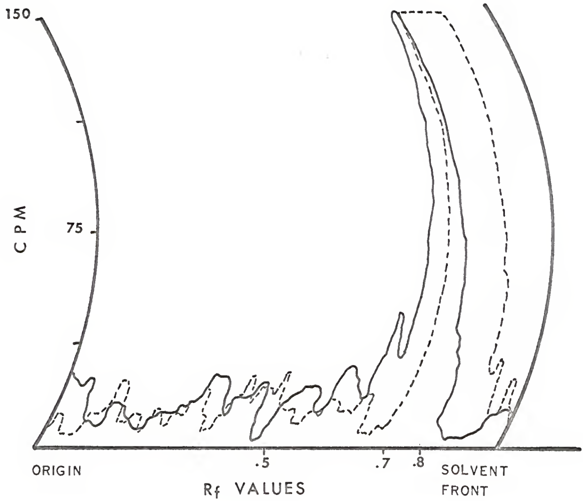


Fig. 18.- Distribution of ^{14}C activity of the Aqueous fraction from 'Valencia' orange leaves. The separation was done by paper chromatography (Whatman No. 1, Isopropanol: Ammonia: water (8:1:1, v/v/v)). (Solid line: 2,4-D-1-^{14}C added to living tissue; dotted line: 2,4-D-1-^{14}C added to extract).

Table 14.- The R_f values of radioactivity components on paper chromatograms of the various fractions from solvent partitioning of 80% ethanolic extracts from 'Pineapple' and 'Valencia' orange leaves.[a]

		R_f			
		'Pineapple'[b]		'Valencia'[c]	
Fractions	Alone	Non-living	Living	Non-living[a]	Living[b]
Hexane	0.72	st[d] 0.72	st	st	st
Ether I	st	st	0.94	st 0.91	st 0.91
Ether II	0.74	st	st 0.92	st 0.8	st
Aqueous	0.72	0.91	0.91	0.8	0.7

[a] See Fig. 1 for the solvent partitioning system.

[b] See Fig. 11 through 14 for treatments and for radioactivity scans of the paper chromatograms.

[c] See Fig. 15 through 18 for treatments and for radioactivity scans of the paper chromatograms.

[d] Evidence of streaking was present on autoradiograms of the paper chromatograms. Exposure time 14 days.

table that detectable radioactive zones occurred only at about the same R_f as that of 2,4-D. Furthermore, not all fractions had detectable regions. Yet, there was considerable radioactivity in the fraction before paper chromatography. This indicates the system of separation and detection was inadequate. This was substantiated further from the autoradiography of the paper chromatograms. All fractions other than the aqueous fraction yielded considerable streaking of radioactive components on the paper chromatograms. Therefore, TLC of the fraction was also conducted.

Thin-Layer Chromatography of the Fractions Obtained by Solvent Partitioning

Using a solvent system similar to the one used by Zenk (94), all fractions from the solvent partitioning of the ethanolic extracts of orange leaves treated with 2,4-D-1-^{14}C or 2,4-D-2-^{14}C prior to, or during, extraction were subjected to TLC. Autoradiograms of the TLC plates were prepared. The R_f values of the various metabolites determined from TLC are shown in Table 15 and were compiled using autoradiograms such as demonstrated in Figs. 19 through 22. From these data it can be seen that in addition to 2,4-D-^{14}C there were a number of radioactive metabolites present in the extract and these were formed within 12 hours. The radioactivity was of the magnitude that only rough estimates could be made of major metabolites.

When the aqueous fractions from the TLC of ethanolic extracts of 'Pineapple' and 'Valencia', treated with 2,4-D-1-^{14}C or 2,4-D-2-^{14}C, were subject to 1N HCl for 3 minutes and the aqueous fraction was

Fig. 19.- Autoradiogram of the TLC of fractions from an ethanolic extract of 'Pineapple' orange leaves treated with 2,4-D-1-^{14}C. The horizontal numbers indicate the following: 1) Hexane fraction; 2) Ether I fraction; 3) Ether II fraction; 4) Aqueous fraction; 5) 2,4-D-1-^{14}C standard. 2,4-D-1-^{14}C uptake-period: 12 hours. Solvent system: Isopropanol:NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.

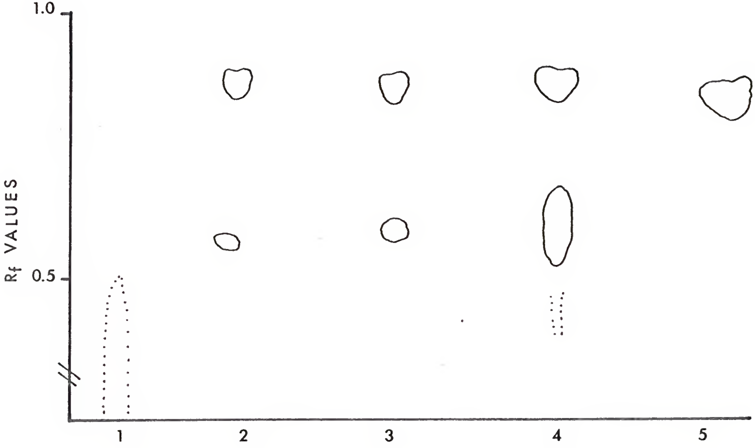

1.0
0.5
R_f VALUES
1
2
3
4
5

Fig. 20.- Autoradiogram of the TLC of fractions from an ethanolic extract of 'Valencia' orange leaves treated with 2,4-D-1-^{14}C. The horizontal numbers indicate the following: 1) Hexane fraction; 2) Ether I fraction; 3) Ether II fraction; 4) Aqueous fraction; 5) 2,4-D-1-^{14}C standard. 2,4-D-1-^{14}C uptake period: 12 hours. Solvent system: Isopropanol:NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.

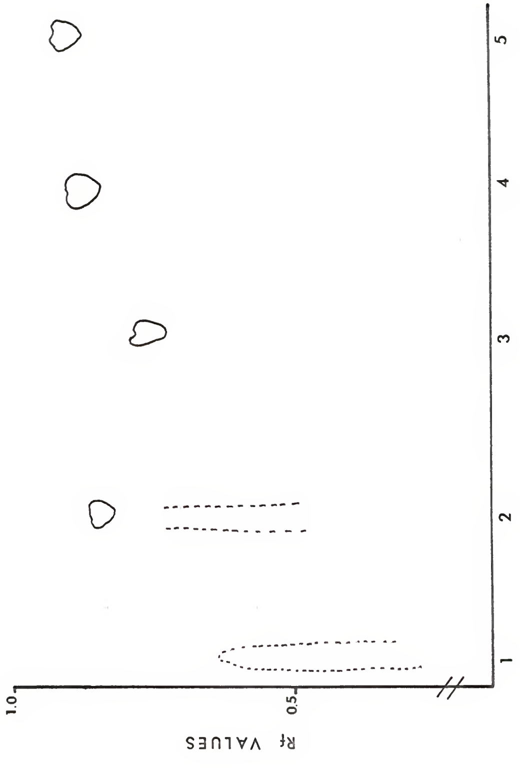

1.0
0.5
Rf VALUES
1
2
3
4
5

Fig. 21.- Autoradiogram of the TLC of fractions from an ethanolic extract of 'Pineapple' orange leaves treated with 2,4-D-2-^{14}C. The horizontal numbers indicate the following: 1) Hexane fraction; 2) Ether I fraction; 3) Ether II fraction; 4) Aqueous fraction; 5) 2,4-D-2-^{14}C standard. 2,4-D-2^{14}C uptake period: 12 hours. Solvent system: Isopropanol:NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.

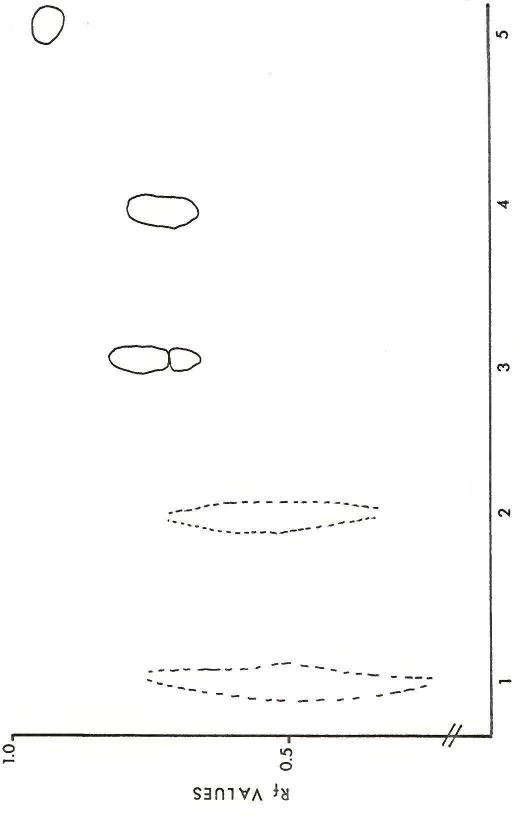

1.0
0.5
R_f VALUES
1
2
3
4
5

Fig. 22.- Autoradiogram of the TLC of fractions from an ethanolic extract of 'Valencia' orange leaves treated with 2,4-D-2-^{14}C. The horizontal numbers indicate the following: 1) Hexane fraction; 2) Ether I fraction; 3) Ether II fraction; 4) Aqueous fraction; 5) 2,4-D-2-^{14}C standard. 2,4-D-2-^{14}C uptake period: 12 hours. Solvent system: Isopropanol:NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.

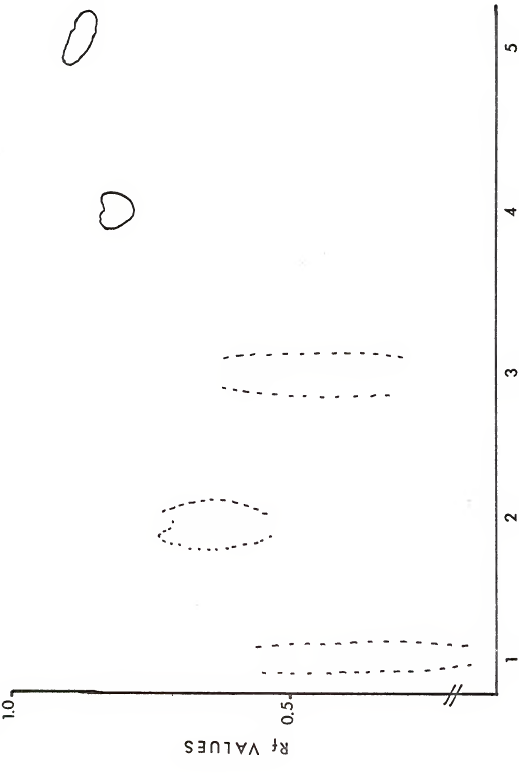

1.0
0.5
R_f VALUES
1
2
3
4
5

Table 15.- The R_f values of spots of radioactivity appearing on TLC of fractions from the solvent partitioning of ethanolic extracts from 'Pineapple' and 'Valencia' orange leaves treated with either 2,4-D-1-^{14}C or 2,4-D-2-^{14}C.[a]

		R_f's of the spots of activity in the different fractions				
Treatment	Variety	Hexane	Ether I	Ether II	Aqueous fraction	2,4-D[b] standard
2,4-D-1-^{14}C	Pineapple	st.[d]	0.52[c] 0.80	0.56[c] 0.83[c]	0.57[c] 0.85[c]	0.84[c]
2,4-D-1-^{14}C	Valencia	st.	0.55[c] 0.86[c]	0.46[c] 0.74[c]	0.86[c]	0.84[c]
2,4-D-2-^{14}C	Pineapple	st.	st.	0.68[c] 0.78[c]	0.67[c]	0.87[c]
2,4-D-2-^{14}C	Valencia	st.	0.56[c]	0.67[c] 0.81[c]	0.81[c]	0.88[c]

[a] See Fig. 1 for the fractionational procedure.

[b] 2,4-D-1-^{14}C chromatographed with the fractions.

[c] Major metabolites.

[d] Evidence of streaking was present on autoradiograms of the paper chromatograms.

Table 16.- Influence of hydrolysis on the radioactive component from the aqueous fractions.[a]

Variety	Labeled 2,4-D used in treatment	R_f values	
		Before hydrolysis	After hydrolysis[b]
Pineapple	2,4-D-1-^{14}C	0.57 - 0.85	0.84
	2,4-D-2-^{14}C	0.67	0.87
Valencia	2,4-D-1-^{14}C	0.86	0.86
	2,4-D-2-^{14}C	0.81	0.93

[a] Radioactivity component from aqueous fractions of TLC.

[b] Hydrolysis was accomplished by 1N HCl.

Fig. 23.- Autoradiogram of the TLC of aqueous fractions and hydrolized aqueous fractions from an ethanolic extract of 'Pineapple' and 'Valencia' orange leaves treated with 2,4-D-1-^{14}C. The horizontal numbers indicate the following: 1) 'Pineapple' aqueous fraction; 2) 'Pineapple' aqueous fractions, hydrolized; 3) 'Valencia' aqueous fraction; 4) 'Valencia' aqueous fractions hydrolized; 5) 2,4-D-1-^{14}C standard. 2,4-D-1-^{14}C uptake period: 12 hours. Solvent system: Isopropanol: NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.

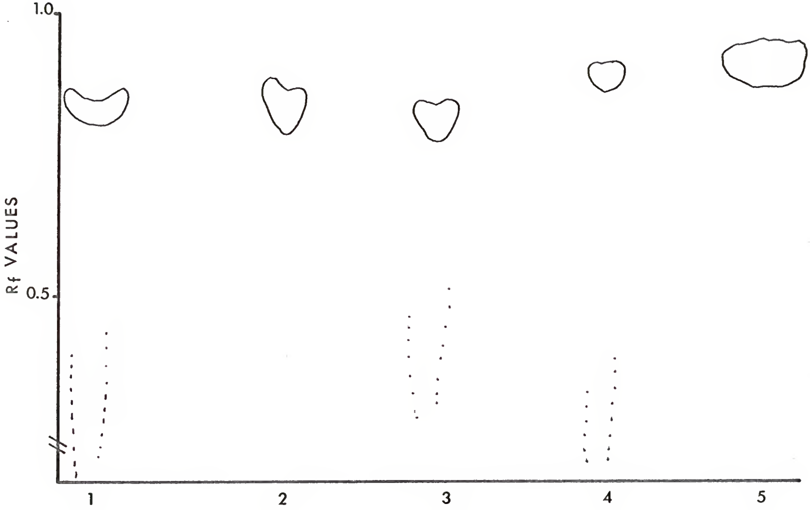
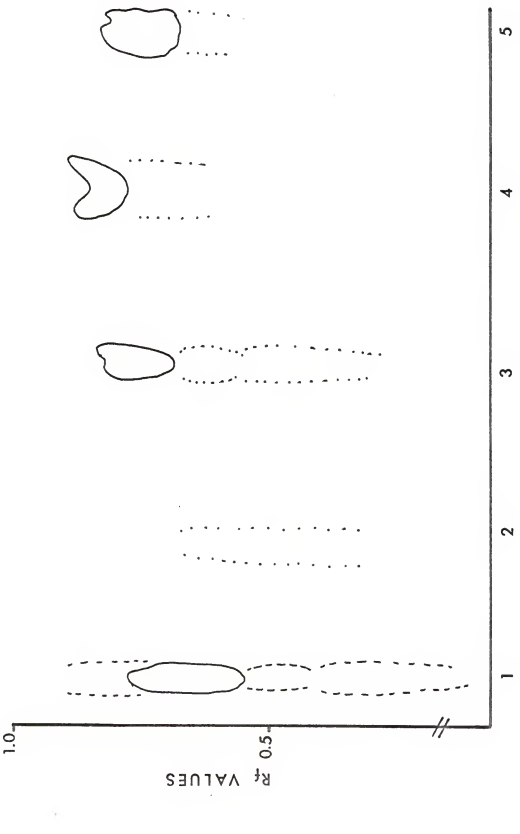

1.0
Rf VALUES
0.5
1
2
3
4
5

Fig. 24.- Autoradiograms of the TLC of aqueous fractions and hydrolized aqueous fractions eluted from paper chromatography of 'Pineapple' and 'Valencia' leaves, previously treated with 2,4-D-1-^{14}C and 2,4-D-2-^{14}C. The horizontal numbers indicate the following: 1) 'Pineapple' treated with 2,4-D-1-^{14}C; 2) 'Valencia' treated with 2,4-D-1-^{14}C; 3) 'Pineapple' treated with 2,4-D-2-^{14}C; 4) 'Valencia' treated with 2,4-D-2-^{14}C; 5) 2,4-D-1-^{14}C standard. Solvent system: Isopropanol:NH_3:H_2O; 8:1:1, v/v/v. X-ray exposure time: 14 days.


1.0
0.5
Rf VALUES
1
2
3
4
5

rechromatographed, all radioactivity was found in a single spot at an R_f that was identical to pure 2,4-D-1-^{14}C (Table 16 and Figs. 23 and 24). This indicated the metabolic compounds were reconverted.

Paper Electrophoretic Separations of the Various Fractions Obtained by Solvent Partition

Fractions from solvent partitioning of the ethanol extract from 'Pineapple' and 'Valencia' orange leaves were subjected to paper electrophoresis. The electrophoretograms were assayed for radioactivity by G-M counting and by autoradiography. Close inspection of the electrophoretograms indicated that there were some metabolites formed by both 'Pineapple' and 'Valencia' leaves treated with 2,4-D-1-^{14}C or 2,4-D-1^{14}C, as can be seen in Figs. 25 through 28. In fraction 1 the 2,4-D-1-^{14}C standard moved toward the anode (b) under the conditions of the experiment, but there was some activity left at the point of application (a). Hexane fractions (2) from both varieties and with both treatments yielded 2 radioactive spots (a and b). Apparently spot (b) corresponded to 2,4-D, but with 'Valencia' there was less resolution. The ether I fraction (3) in all treatments with 'Pineapple' yielded 2 distinctive spots (a and b), here again (b) would seem to be 2,4-D; the same was true with 'Valencia' when treated with 2,4-D-1-^{14}C. When 'Valencia' was treated with 2,4-D-2-^{14}C, 5 radioactive spots were evident, 3 of them (a, b and c) moved to the cathode, and of the other 2 one remained at the point of application and the other migrated toward the anode (e).

The ether II fraction (4) from 'Pineapple' treated with 2,4-D-1-^{14}C gave 3 different radioactive zones (b, c, d), and some activity was left

Fig. 25.- Autoradiogram of the electrophoretic separation of ^{14}C metabolites resulting from 2,4-D-1-^{14}C uptake by 'Pineapple' orange leaves. The horizontal numbers indicate the following: 1) 2,4-D-1-^{14}C standard; 2) Hexane fraction; 3) Ether I fraction; 4) Ether II fraction; 5) Aqueous fraction. Developed for 4 hours at 500 volts in 0.04 acetic acid buffer (pH 2.5). (Dotted line indicates a diffuse boundary).

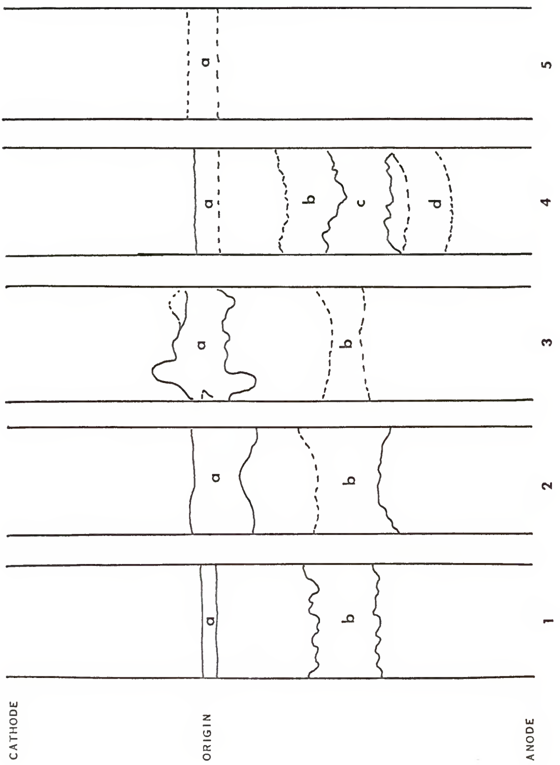

CATHODE
ORIGIN
ANODE
1
a
b
2
a
b
3
a
b
4
a
b
c
d
5
a

Fig. 26.- Autoradiogram of the electrophoretic separation of ^{14}C metabolites resulting from 2,4-D-1-^{14}C uptake by 'Valencia' orange leaves. The horizontal numbers indicate the following: 1) 2,4-D-1-^{14}C standard; 2) Hexane fraction: 3) Ether I fraction; 4) Ether II fraction; 5) Aqueous fraction. Developed for 4 hours at 500 volts in 0.04 acetic acid buffer, (pH 2.5). (Dotted line indicates a diffuse boundary).

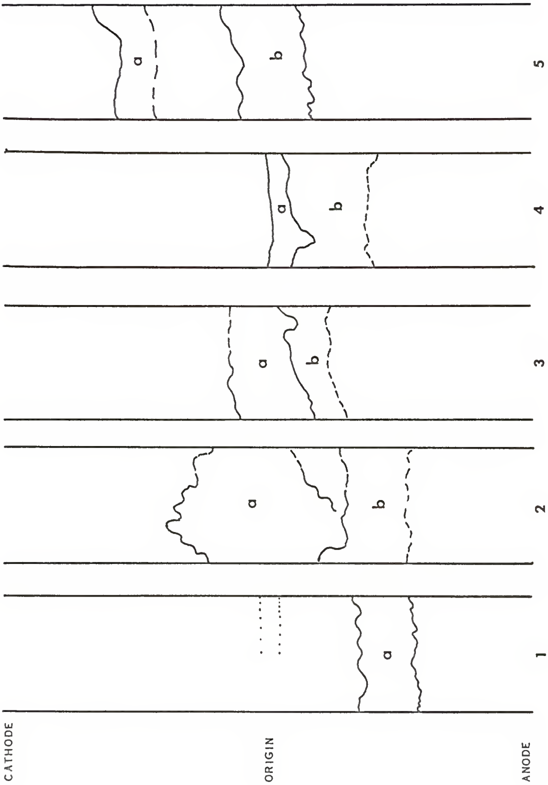

CATHODE
ORIGIN
ANODE
1
a
2
a
b
3
a
b
4
a
b
5
a
b

Fig. 27.- Autoradiogram of the electrophoretic separation of ^{14}C metabolites resulting from 2,4-D-2-^{14}C uptake by 'Pineapple' orange leaves. The horizontal numbers indicate the following: 1) 2,4-D-2-^{14}C standard; 2) Hexane fraction; 3) Ether I fraction; 4) Ether II fraction and 5) Aqueous fraction. Developed for 4 hours at 500 volts in 0.04 acetic acid buffer (pH 2.5). (Dotted line indicates a diffuse boundary).

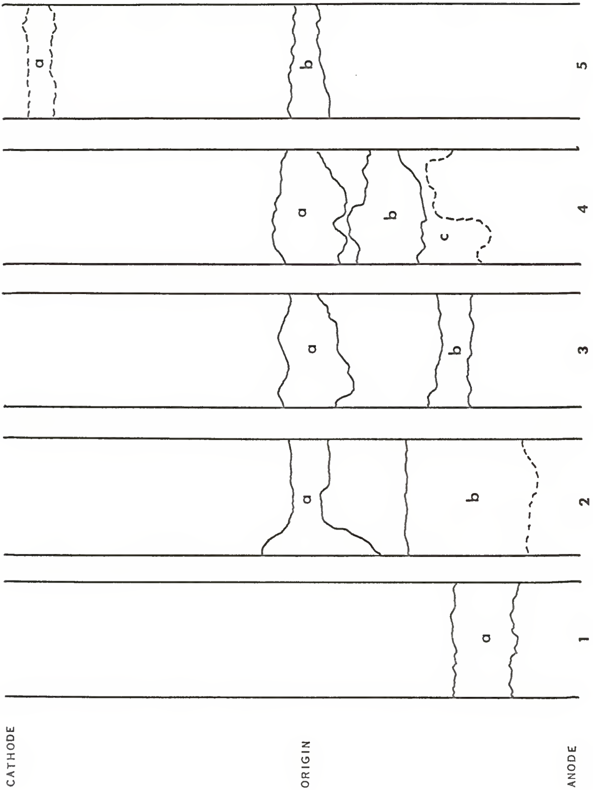
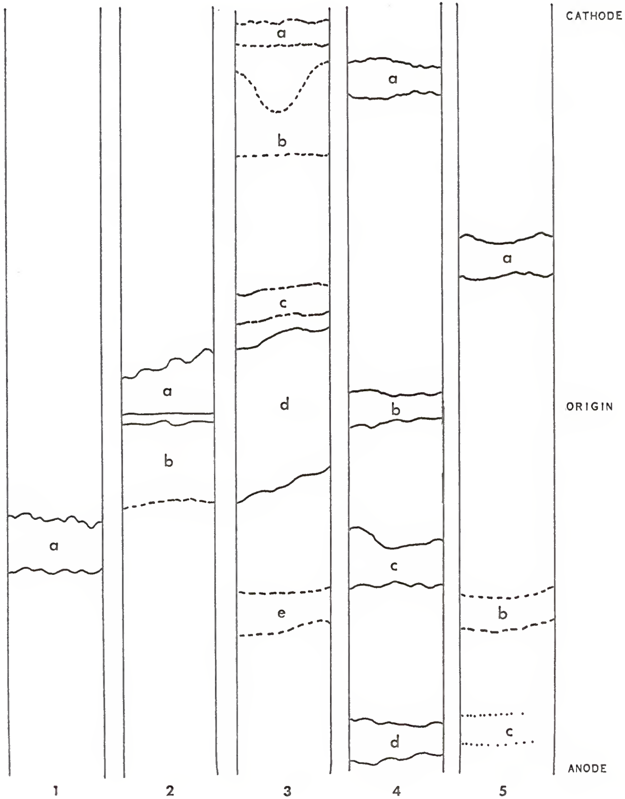

CATHODE
ORIGIN
ANODE
1
a
2
a
b
3
a
b
4
a
b
c
5
a
b

Fig. 28.- Autoradiogram of the electrophoretic separation of ^{14}C metabolites resulting from 2,4-D-2-^{14}C uptake by 'Valencia' orange leaves. The horizontal numbers indicate the following: 1) 2,4-D-2-^{14}C; 2) Hexane fraction; 3) Ether I fraction; 4) Ether II fraction and 5) Aqueous fraction. Developed for 4 hours at 500 volts in 0.04 acetic acid buffer (pH 2.5). (Dotted line indicates a diffuse boundary).


CATHODE
ORIGIN
ANODE
a
b
c
d
e
1
2
3
4
5

at the point of application (a). For 'Pineapple' treated with 2,4-D-2-^{14}C the pattern were almost the same, but apparently there was a lack of resolution. In 'Valencia' treated with 2,4-D-1-^{14}C there were 2 spots (a and b) in the ether II fraction. In the ether II fraction from 'Valencia' 2,4-D-2-^{14}C treated, there were 4 distinct radioactive spots, one moved to the cathode (a), one remained at the point of application (b), and 2 migrated toward the anode (c) (d). The aqueous fraction (5) from 'Pineapple' showed just one spot at the point of application when treated with 2,4-D-2-^{14}C, (Fig. 26) and 2 spots (a and b) when treated with 2,4-D-2-^{14}C (Fig. 28). Of these, one (a) moved toward the anode and one (b) remained at the origin. Fraction 5 from 'Valencia', 2,4-D-1-^{14}C treated (Fig. 27), showed 2 distinctive zones (a) (b), one was at the origin (b) and the other migrated toward the cathode (a). When 'Valencia' was treated with 2,4-D-2-^{14}C (Fig. 29), the aqueous fraction (5) contained 3 spots, one moved toward the cathode (a), and 2 toward the anode (b) (c). The zone (b) nearest the origin of the latter was apparently 2,4-D.

In general, the results indicated that 'Valencia' gave more distinguishable radioactive components than 'Pineapple', especially when treated with 2,4-D-2-^{14}C.

Influence of 2,4-D on Glucose Metabolism

The first series of tests were conducted using material from 'Pineapple' and 'Valencia' leaves that were treated with or without 2,4-D prior to removing the leaf discs. Using Warburg flasks, the $^{14}CO_2$ was trapped at the same time O_2 uptake was being determined. The data on

Table 17.- Effect of 2,4-D treatment on the rate of respiration of leaf discs of 'Pineapple' orange.[a]

		μl of O_2[b]		
Expt.	Treatment	1 hr.	2 hrs.	3 hrs.
1	Buffer	73.6	124.4	300.0
	Buffer + 2,4-D	77.6	206.0	611.6
2	Buffer	155.6	292.0	460.6
	Buffer + 2,4-D	210.8	625.2	689.6
3	Buffer	78.8	254.8	346.8
	Buffer + 2,4-D	69.2	406.0	513.6
4	Buffer	128.8	227.6	389.6
	Buffer + 2,4-D	218.8	339.2	853.2
5	Buffer	68.8	282.4	296.4
	Buffer + 2,4-D	-	-	-
6	Buffer	71.6	218.8	421.2
	Buffer + 2,4-D	203.2	349.2	527.6
7	Buffer	147.6	203.2	282.4
	Buffer + 2,4-D	202.0	214.4	571.2
8	Buffer	162.4	321.6	493.6
	Buffer + 2,4-D	175.6	319.2	498.0

[a] 2,4-D and buffer solutions applied by petiole uptake 12 hours before the leaf discs were punched and placed in glucose-1-^{14}C or glucose-6-^{14}C.

[b] μl of oxygen utilized per hour per gm fresh weight of tissue.

Table 18.- Effect of 2,4-D treatment on the rate of respiration of leaf discs of 'Valencia' orange.[a]

		μl of O_2[b]		
Expt.	Treatment	1 hr.	2 hrs.	3 hrs.
1	Buffer	70.4	230.0	402.4
	Buffer + 2,4-D	123.6	254.0	410.0
2	Buffer	108.1	307.6	476.4
	Buffer + 2,4-D	139.2	350.0	510.8
3	Buffer	136.4	351.6	511.2
	Buffer + 2,4-D	122.0	340.0	472.0
4	Buffer	191.6	458.8	495.2
	Buffer + 2,4-D	175.6	-	-
5	Buffer	55.2	218.0	392.8
	Buffer + 2,4-D	118.4	398.8	465.6
6	Buffer	140.4	168.8	470.0
	Buffer + 2,4-D	170.4	412.1	439.6
7	Buffer	232.4	465.6	527.6
	Buffer + 2,4-D	143.2	395.2	481.2
8	Buffer	279.2	510.0	522.8
	Buffer + 2,4-D	185.6	430.0	485.2

[a] 2,4-D and buffer solutions applied by petiole uptake 12 hours before the leaf discs were punched and placed in glucose-1-^{14}C or glucose-6-^{14}C.

[b] μl of oxygen utilized per hour per gm fresh weight of tissue.

Table 19.- Effect of 2,4-D treatment on the rate of respiration of leaf discs of 'Pineapple' and 'Valencia' oranges.[a]

		μl of O_2[b,c]		
Variety	Treatment	1 hr.	2 hrs.	3 hrs.
Pineapple	Buffer	111.4	215.7	373.8
Pineapple	Buffer + 2,4-D	116.1	351.4	609.3
Valencia	Buffer	151.3	338.8	474.8
Valencia	Buffer + 2,4-D	147.4	336.8	439.8

[a] See Tables 15 and 16 for method of treatment.

[b] Average of 8 replications.

[c] μl of oxygen utilized per hour per gm fresh weight of tissue. The values are accumulative totals for the times indicated after the start of glucose uptake.

Table 20.- Effect of 2,4-D treatment of 'Pineapple' and 'Valencia' orange leaves on the metabolism of glucose-1-^{14}C and glucose-6-^{14}C.

Expt.	Variety	Treatment[a]	Per cent of ^{14}C added recovered as $^{14}CO_2$[b]		
			Glucose-1-^{14}C	Glucose-6-^{14}C	C_6/C_1
1	Pineapple	Buffer	3.36	1.28	0.38
	Pineapple	Buffer + 2,4-D	2.30	2.56	1.11
2	Pineapple	Buffer	5.60	2.22	0.40
	Pineapple	Buffer + 2,4-D	8.60	2.73	0.31
3	Pineapple	Buffer	3.78	1.98	0.52
	Pineapple	Buffer + 2,4-D	3.44	1.65	0.48
4	Pineapple	Buffer	4.94	2.22	0.45
	Pineapple	Buffer + 2,4-D	5.80	1.95	0.34
5	Valencia	Buffer	2.83	1.88	0.66
	Valencia	Buffer + 2,4-D	2.85	2.03	0.71
6	Valencia	Buffer	3.22	2.14	0.66
	Valencia	Buffer + 2,4-D	3.02	1.99	0.66
7	Valencia	Buffer	2.97	1.86	0.63
	Valencia	Buffer + 2,4-D	2.52	2.21	0.87
8	Valencia	Buffer	2.87	2.13	0.60
	Valencia	Buffer + 2,4-D	3.07	2.11	0.69

[a] 2,4-D and buffer solutions applied by petiole-uptake 12 hours before the leaf discs were cut and placed in the radioactive glucose.

[b] $^{14}CO_2$ was trapped in 10% KOH in the center well of Warburg flasks with the explants in the outer chamber. Reaction stopped with 1N H_2SO_4.

Table 21.- Effect of 2,4-D treatment of 'Pineapple' and 'Valencia' orange leaves on the metabolism of glucose-1-^{14}C and glucose-6-^{14}C.[a,b]

		$^{14}CO_2$ evolution (cpm)				C_6/C_1			
Variety	Treatment	1 hr.	2 hrs.	3 hrs.	4 hrs.	1 hr.	2 hrs.	3 hrs.	4 hrs.
Pineapple	G-1-^{14}C + 2,4-D	2,800	6,750	11,300	17,300	0.75	0.68	0.59	0.57
Pineapple	G-6-^{14}C + 2,4-D	2,111	4,588	6,699	9,866				
Pineapple	G-1-^{14}C + buffer	2,650	7,800	24,000	36,250	0.57	0.53	0.72	0.64
Pineapple	G-6-^{14}C + buffer	1,502	4,141	17,904	23,345				
Valencia	G-1-^{14}C + 2,4-D	17,150	25,450	32,750	43,450	0.1	0.16	0.31	0.64
Valencia	G-6-^{14}C + 2,4-D	1,786	3,979	10,150	14,980				
Valencia	G-1-^{14}C + buffer	2,000	33,000	41,050	54,800	1.44	0.25	0.54	0.49
Valencia	G-6-^{14}C + buffer	2,883	8,364	22,005	26,674				

[a] 2,4-D and radioactive glucose applied at the same time by petiole uptake.

[b] Both glucose solutions yielded approximately 12,000,000 cpm with the sample system used to assay for $^{14}CO_2$.

O_2 uptake are presented in Table 17 for 'Pineapple' leaf discs, Table 18 for 'Valencia' leaf discs and in Table 19 a comparison is made of the means of the 8 experiments with the 2 varieties. There was little difference between the oxygen consumption of 2,4-D treated and control leaf discs during the first hour after treatment (13 hours after the initial uptake of 2,4-D, 1 hour after placing discs in the glucose solution). 'Pineapple' leaf discs treated with 2,4-D increased the rate of O_2 consumption, 2 and 3 hours after treatment. This was in contrast to 'Valencia' leaf discs where buffer and 2,4-D treated leaf discs had very similar O_2 uptake patterns.

Table 20 contains the data of the % of added ^{14}C recovered as $^{14}CO_2$ from Glucose-1-^{14}C (G-1-^{14}C) and Glucose-6-^{14}C (G-6-^{14}C) and the C_6/C_1 ratios obtained from these yields. Comparisons were made for 'Pineapple' and 'Valencia' leaves of buffer treated and 2,4-D treated leaf discs. With one exception (Expt. 1) the C_6/C_1 ratio from 'Pineapple' orange leaf was lowered as a result of 2,4-D treatment, the C_6/C_1 ratio from 'Valencia' orange leaf discs was slightly raised (Expts. 5, 7 and 8) or remained unchanged (Expt. 6). The total yield of $^{14}CO_2$ from either G-6-^{14}C or G-1-^{14}C was not greatly affected by 2,4-D treatment.

Humphreys and Dugger (49) indicated that the experimental time period used (3.5 hours) in their experiment was close to the maximum allowable if any significant C_6/C_1 ratio are to be obtained. For this reason, another experiment was devised to determine the effect of 2,4-D with time on 'Pineapple' and 'Valencia' orange leaves on the metabolism of radioactive glucose. To accomplish this, glucose and 2,4-D were applied simultaneously by petiole uptake and the entire leaves were placed in a closed system to trap the $^{14}CO_2$ evolved. With this closed system, the data of Table 21 were obtained on the % of added ^{14}C recovered as $^{14}CO_2$ from G-1-^{14}C and G-6-^{14}C every hour for 4 hours. The ratios obtained were compared for both 'Pineapple' and 'Valencia' orange leaves from buffer treated and 2,4-D treated leaves.

To aid in comparing the CO_2 patterns, Fig. 29 portrays the effect of 2,4-D on the $^{14}CO_2$ evolved from metabolism of glucose-1-^{14}C by 'Pineapple' and 'Valencia' leaves; Fig. 30 portrays the effect of 2,4-D on the $^{14}CO_2$ evolved from the metabolism of glucose-6-^{14}C by both varieties and Fig. 31 portrays the influence of 2,4-D on the ratio of $^{14}CO_2$ evolved from the metabolism of G-1-^{14}C and G-6-^{14}C by the 2 varieties. The latter demonstrates that there was a decrease in the C_6/C_1 ratio in time from 2,4-D treated 'Pineapple' leaves, and that there was an increase in time of the C_6/C_1 ratio from buffer treated 'Pineapple' leaves. 'Valencia' leaves treated with 2,4-D gave very low C_6/C_1 ratios, which slightly increases with time. The same was true for buffer treated 'Valencia' leaves.

The C_6/C_1 ratio value obtained from 'Valencia' leaves, buffer treated, at 1 hour apparently was an exception, this high value could be due to pool levels of glucose in the tissue which could cause large sources of error at the start of this type of experiment. Furthermore, initial uptake and adding to pools would differ considerably initially.

From both varieties the yield of $^{14}CO_2$ from G-1-^{14}C was significantly decreased by 2,4-D treatment (Fig. 29). Similarly the yield of $^{14}CO_2$ from G-6-^{14}C (Fig. 30) was also decreased by 2,4-D treatment of

Fig. 29.- Effect of 2,4-D treatment of 'Pineapple' and 'Valencia' orange leaves on the metabolism of Glucose-1-^{14}C (see Table 21 for parameters).

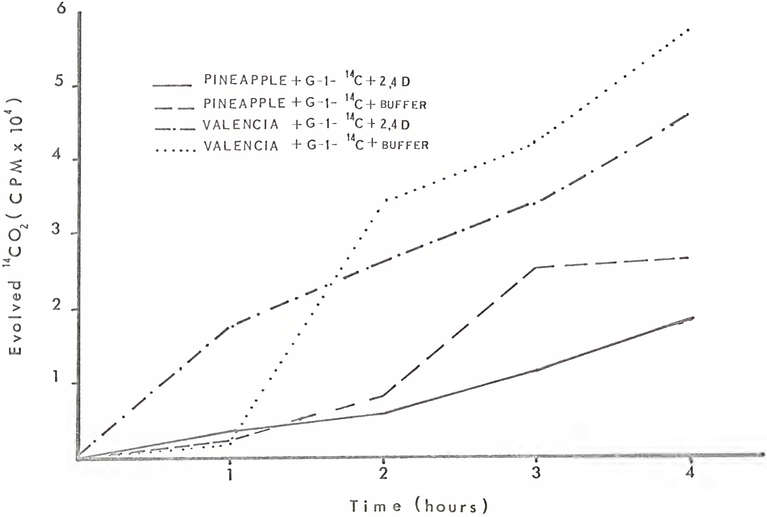

PINEAPPLE + G-1- ^{14}C + 2,4 D
PINEAPPLE + G-1- ^{14}C + BUFFER
VALENCIA + G-1- ^{14}C + 2,4 D
VALENCIA + G-1- ^{14}C + BUFFER
Evolved $^{14}CO_2$ (C P M x 10^4)
6
5
4
3
2
1
1
2
3
4
Time (hours)

Fig. 30.- Effect of 2,4-D treatment of 'Pineapple' and 'Valencia' orange leaves on the metabolism of Glucose-6-^{14}C (see Table 21 for parameters).

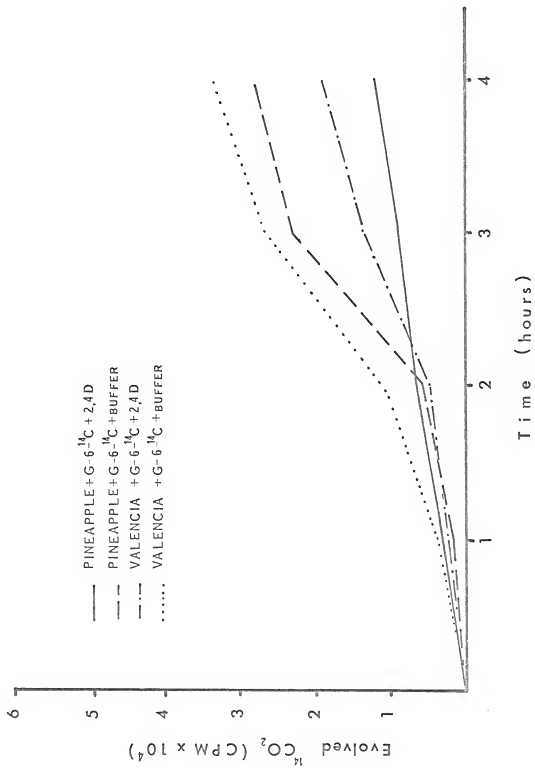
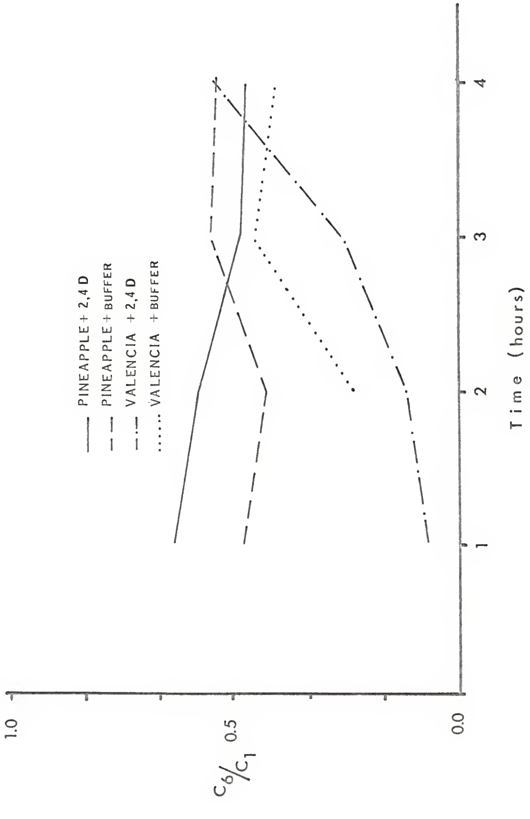

PINEAPPLE + G-6-^{14}C + 2,4D
PINEAPPLE + G-6-^{14}C + BUFFER
VALENCIA + G-6-^{14}C + 2,4D
VALENCIA + G-6-^{14}C + BUFFER
Evolved ^{14}CO$_2$ (C PM x 10^4)
1
2
3
4
5
6
Time (hours)
1
2
3
4

Fig. 31.- Effect of 2,4-D treatment of 'Pineapple' and 'Valencia' orange leaves on the rate of the release of $^{14}CO_2$ by metabolism of Glucose-1-^{14}C, and Glucose-6-^{14}C (see Table 21 for parameters).


PINEAPPLE + 2,4 D
PINEAPPLE + BUFFER
VALENCIA + 2,4 D
VALENCIA + BUFFER
C_6/C_1
1.0
0.5
0.0
1
2
3
4
Time (hours)

both varieties. These experiments clearly indicate that 2,4-D was getting into the tissues rapidly, and it was affecting metabolism of a substrate material. This is in addition to the metabolism of 2,4-D *per se*.

DISCUSSION

From previous works (40, 71, 74), it has been shown that abscission of fruits and leaves of 'Pineapple' and 'Valencia' orange varieties are different when subjected to identical 2,4-D treatments; however, the data presented here demonstrate that 2,4-D was absorbed and translocated almost at the same rate in the 2 varieties. This was evident when treated leaves were extracted with ethanol, and the radioactivity determined. Autoradiograms of leaves did not have sufficient definition to yield much information on uptake and translocation, probably because of the rather thick leaf which absorbed the weak Beta particles emitted by ^{14}C decay. Even with petiole-uptake tests where there was a greater quantity of 2,4-D-1-^{14}C absorbed into the tissues than through the surface of the leaf, there was poor definition on the leaf autoradiograms. This demonstrated that the autoradiogram method with the entire leaf was not suitable for this problem.

Both varieties were found to be capable of metabolizing 2,4-D-1-^{14}C and 2,4-D-2-^{14}C with the subsequent release of $^{14}CO_2$. The evolution of $^{14}CO_2$ occurred at about the same rate from both varieties, and there was no evidence of the induction of an enzymic system since the release of $^{14}CO_2$ was linear with time.

The fact that $^{14}CO_2$ production was 4 times as much from 2,4-D-1-^{14}C as from 2,4-D-2-^{14}C indicated that there was an accumulation of intermediates containing the methylene carbon to a much greater extent than the carboxyl carbon of the 2,4-D molecule, which agrees with previous works with other tissues on metabolism of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C (60, 90, 91), even though the reasons for this difference are not clear. However, when leaves metabolize acetate-1-^{14}C or acetate-2-^{14}C the evolution of ^{14}C from the carboxyl carbon has been as much as 4 times as fast (34, 35, 36, 51). Apparently, the methylenic carbons were entering the metabolic pool and used in synthetic reactions.

When pure 2,4-D-1-^{14}C was subjected to solvent-partitioning fractionation, the radioactivity remained mostly in the aqueous fraction. The addition of ethanol-killed tissue to pure 2,4-D-1-^{14}C distorted the fractionational system. This distortion of the solvent partitioning system should be examined very carefully in relation to previous works (1, 4, 45, 46, 54). Andreae and Good (1), Hay and Thimann (45, 46) and others (54, 83) have used a similar partitioning system to fractionate extracts from 2,4-D treated tissues of different plant species. They have reported the presence of metabolites in various solvent fractions by comparing R_f values. According to the results presented here the fractionational system is altered when extracted components from killed tissue are present. Thus, some caution must be exercised when considering components in the various solvent fractions and when considering R_f of the components in a single fraction.

With 'Pineapple' leaves the tissue reactions caused a greater percentage of radioactivity to occur in the hexane and ether fractions, with a concomitant decrease in the aqueous fraction. With 'Valencia' leaves, the tissue reactions caused an increase in radioactivity of the hexane fraction with a concomitant decrease in the ether II and aqueous fraction.

This difference in distribution indicated that 2,4-D was metabolized in the tissues, and that 'Pineapple' and 'Valencia' orange tissues metabolized 2,4-D to different products.

Fractionation of the extracts from both varieties by TLC and electrophoresis demonstrated the presence of free 2,4-D and a number of 2,4-D products, but there were a greater number of metabolites present from 'Valencia' tissue than from 'Pineapple' tissue. It would seem logical that 'Valencia' may degrade more of the 2,4-D to products that do not subsequently become converted back to 2,4-D. Thus, a greater quantity of the 2,4-D is permanently detoxified.

Previous workers have shown that complexes of unknown composition are produced in the metabolism of 2,4-D by the plant (1, 4, 16, 54). Jaworski and Butts (54) suggested that one of their unidentified compounds might be a glycoside containing 2,4-D as the aglycon. Andreae and Good (1) identified 2,4-dichlorophenoxyacetoaspartic acid as a metabolic product of 2,4-D. Also Zenk (93) reported that a second detoxification mechanism of aromatic acids occurred in plants, that is the conjugation with glucose. Later, Sudi (83) found that carboxylic acids (2,4-D included) had the ability to induce the formation of both IAA-aspartate and NAA-aspartate. Complexes with sugars, amino acids or other products by the formation of either an ethereal oxygen or an ester oxygen bridge will yield free 2,4-D again by catalytic or non-catalytic hydrolysis.

Although, there was no direct answer as to how 2,4-D prevented abscission in orange leaves, certain observations can be made that have merit. As shown by many investigators (12, 18, 58, 68), natural leaf abscission is related to aging; and associated with the final stages of aging which leads to senescence, is a lowered respiratory capacity of leaves when compared to healthy, mature leaves. Possibly, a shift in the pattern of glucose metabolism is associated with this lowered respiratory capacity (42). It was suggested that the PPP metabolism of glucose is associated with aging (42). In other words, as the organ matures, glucose metabolism via PPP becomes increasingly more prominent than metabolism by EMP (42). Relating these observations with those that 2,4-D has been found to increase respiration without changing the RQ (49, 50), or increasing the carbohydrate level in the tissue (49, 50) and that 2,4-D caused an increase in the amount of glucose catabolized via the EMP, and it seemed that the influence of 2,4-D on glucose metabolism of citrus leaves could be related to abscission.

Data obtained herein showed that respiration of 'Pineapple' leaves was increased when treated with 2,4-D. This was in contrast to 'Valencia' leaves which did not respond to 2,4-D treatment by an increase in respiration. Also, it was shown that there was a shift in glucose metabolism to more being metabolized by the EMP by 'Pineapple' orange leaves. This was not evident for 'Valencia' orange leaves.

From these observations, it was concluded that 2,4-D was affecting glucose metabolism of 'Pineapple' tissues to a much greater extent than the glucose metabolism of 'Valencia' tissues, and that this was possibly involved in the inhibition of abscission of 'Pineapple' leaves.

SUMMARY AND CONCLUSIONS

Investigations were made to determine the patterns of uptake, translocation and metabolism of labeled 2,4-D in 'Pineapple' and 'Valencia' sweet orange leaves, and its possible effect on abscission. The following observations were made:

1. Both 'Pineapple' and 'Valencia' orange leaves absorbed and translocated 2,4-D fairly rapidly and almost at an identical rate, as determined by following uptake of 2,4-D-1-^{14}C.
2. Even when large quantities of 2,4-D-1-^{14}C were present in the tissues as shown by extraction, the leaf autoradiograms gave poor definition, demonstrating that the autoradiography method with the entire citrus leaf was not suitable for this problem.
3. 'Pineapple' and 'Valencia' orange leaves were found to metabolize 2,4-D-1-^{14}C and 2,4-D-2-^{14}C, with the subsequent release of $^{14}CO_2$.
4. The evolution of $^{14}CO_2$ was linear with time with both varieties; thus, there was no evidence of an inductive enzymic system.
5. There was production of $^{14}CO_2$ arising from metabolism of 2,4-D-1-^{14}C that was 4 times greater than the production of $^{14}CO_2$ coming from 2,4-D-2-^{14}C, indicating the accumulation of intermediates containing the methylene carbon to a much greater extent than the carboxyl carbon of the 2,4-D molecule.
6. Addition of pure 2,4-D-1-^{14}C to ethanol-killed tissue distorted

the solvent partitioning system of fractionation.

7. It was found that there were differences in distribution of radioactivity in the different fractions after 2,4-D was exposed to living tissue for 24 hours, indicating that 2,4-D was metabolized by the tissues.
8. 'Pineapple' and 'Valencia' orange leaves metabolized 2,4-D to different products with a greater number of metabolites present in extracts from 'Valencia' than from 'Pineapple'.
9. 'Valencia' orange leaves degraded more of the 2,4-D; thus a greater quantity was possibly permanently detoxified.
10. The rate of respiration of 'Pineapple' leaves was increased by 2,4-D treatment, in contrast to the respiration of 'Valencia' leaves which neither increased nor decreased.
11. There was a shift in glucose metabolism in 'Pineapple' leaves with more glucose being metabolized by the EMP. This shift was not apparent with 'Valencia' leaves. It was concluded that 2,4-D was affecting glucose metabolism of 'Pineapple' tissues to a greater extent than that of 'Valencia' tissues, and this effect on metabolism in luences the inhibition of abscission of 'Pineapple' leaves. The greater influence of 2,4-D on metabolism of 'Pineapple' leaves may be due to more free 2,4-D present for a longer period of time in 'Pineapple' leaves since 'Valencia' leaves produces a greater number of metabolites.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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